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Synovial fluid analysis in healthy green turtles *Chelonia mydas* in Taiwan

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ABSTRACT: Septic arthritis is a frustrating disease in sea turtle rehabilitation because of its unclear pathogenesis, delayed onset during rehabilitation, long-term treatment requirements, and potentially poor prognosis. Radiography, blood cultures, and arthrocentesis have been used as diagnostic tools for suspected cases. However, there is currently a lack of data on the characteristics of synovial fluid in healthy sea turtles. To establish reference data for synovial fluid in sea turtles, we enrolled 14 green turtles *Chelonia mydas* rescued between 2019 and 2022 from 3 facilities using the following inclusion criteria: normal attitude and appetite, normal motor functions of the 4 limbs, no joint swelling, and no ongoing use of antibiotics for at least 1 mo. Bacterial cultures of blood and synovial fluid from the shoulder joints of these turtles were obtained and a qualitative analysis of the synovial fluid was performed. The results revealed bacterial culture-negative blood and synovial fluids at 37°C. Most characteristics of normal synovial fluid in green turtles, such as being transparent, colorless, and able to create a strand of over 2.5 cm by being pulled with a needle in viscosity trials, as well as the cytology of the normal synovial fluids being dominated by histiocytes and synovial lining cells, lymphocytes, and occasionally a few heterophils or erythrocytes were similar to those in mammals. This study provides information on the normal synovial fluid characteristics of green turtles in Taiwan, which may be beneficial for the diagnosis of joint diseases in sea turtles.

KEY WORDS: Sea turtles · Septic arthritis · Synovial fluid analysis · Blood culture · Arthrocentesis · Osteomyelitis

1. INTRODUCTION

There are 7 species of sea turtles worldwide, all of which are included in the Red List of the International Union for Conservation of Nature (IUCN 2023). Among them, 5 species, namely green *Chelonia mydas*, hawksbill *Eretmochelys imbricata*, olive ridley *Lepidochelys olivacea*, leatherback *Dermochelys coriacea*, and loggerhead sea turtles *Caretta caretta*, have been observed along the coastline of Taiwan (Kuo et al. 2017, Cheng et al. 2019). Taiwan serves as an important habitat and nesting site for green turtles (Cheng et al. 2019). Similar to sea turtles around the world, sea turtles in Taiwan are exposed to several threats, such as fishery interactions, watercraft injuries, ocean pollution, and diseases (Shigenaka et al. 2021). Given these challenges, facilitating opportunities for biological and veterinary research using data from sea turtles in Taiwan is invaluable for the conservation of these species.

Among the diseases that affect sea turtle rehabilitation, osteolytic arthritis is one of the most challenging

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(Harms et al. 2002, Greer et al. 2003, Raidal et al. 2006, Guthrie et al. 2010, Cruciani et al. 2019, Powell et al. 2021). Frustration arises primarily because of several factors. First, the pathogenesis remains unclear. Many cases of arthritis develop weeks to months after stranding in turtles undergoing prolonged rehabilitation (Harms et al. 2002, Greer et al. 2003, Solano et al. 2008, Innis et al. 2014, Cruciani et al. 2019, Powell et al. 2021) while other cases are pre-existing at the time of stranding (Solano et al. 2008, Guthrie et al. 2010, Cruciani et al. 2019). It has been hypothesized that the infection in these cases was caused by bacteria in the gastrointestinal flora (Ogden et al. 1981). Under chronic stress conditions, bacteria can invade the bloodstream and cause bacteremia. Subsequently, these bacteria may enter the joints via a hematogenous route, leading to septic arthritis or osteomyelitis (Ogden et al. 1981). Second, long-term treatment is often required. Various reports, along with our experience, have revealed that treatment lasts from several months to over a year until full recovery (Harms et al. 2002, Guthrie et al. 2010, Powell et al. 2021). A recent retrospective study of osteomyelitis in cold-stunned Kemp's ridley sea turtles *L. kempii* revealed a median hospitalization duration of 256 d, with either conservative treatment or a combination of medical and surgical management before the animals were released (Powell et al. 2021). Third, while the majority of cases can be successfully resolved, some cases have a poor outcome despite intensive treatments (Greer et al. 2003, Raidal et al. 2006, Guthrie et al. 2010, Powell et al. 2021).

Bacterial pathogens commonly associated with infections in sea turtles include *Mycobacterium chelonae*, *Enterococcus* spp., *Nocardia* spp., *Streptococcus* spp., *Corynebacterium* spp., *Actinobacter wolffi*, *Staphylococcus* spp., *Morganella morganii*, *Serratia marcescens*, *Citrobacter braakii*, *Acinetobacter haemolyticus*, *Escherichia coli*, *Pseudomonas* spp., and *Vibrio alginolyticus*. These pathogens have been documented in several studies (Harms et al. 2002, Greer et al. 2003, Solano et al. 2008, Guthrie et al. 2010, Innis et al. 2014, Tsai et al. 2019, Cruciani et al. 2019, Powell et al. 2021), and periarticular swelling and lameness are the most common clinical presentations among infected individuals (Cruciani et al. 2019, Powell et al. 2021). Nonspecific clinical signs, including hyporexia, lethargy, weight loss, and buoyancy disorder, can also be noted, whereas a few cases can be incidentally diagnosed using radiographic lesions (Cruciani et al. 2019). According to previously published studies, the joints of the forelimbs have demonstrated a higher occurrence of pathologies than those of the hind limbs (Harms et al. 2002, Greer et al. 2003, Raidal et al. 2006, Solano et al. 2008, Guthrie et al. 2010, Innis et al. 2014, Cruciani et al. 2019, Powell et al. 2021). The frequency of reported cases among various joints is as follows: shoulder joints, 3 individuals (5 joints) (Guthrie et al. 2010, Cruciani et al. 2019); elbow joints, 11 individuals (14 joints) (Greer et al. 2003, Cruciani et al. 2019); carpal, metacarpal, and interphalangeal joints, 5 individuals (24 joints) (Harms et al. 2002, Solano et al. 2008, Cruciani et al. 2019); and stifle joints, 3 individuals (4 joints) (Cruciani et al. 2019). Notably, a substantial percentage of cases showed multiple joint involvement (Powell et al. 2021). A recent 10 yr retrospective study of osteomyelitis in Kemp's ridley sea turtles revealed radiographic findings that osteolytic lesions most commonly appeared on the humeral bones, although it did not indicate whether the proximal or distal joints were affected (Powell et al. 2021).

Radiographs, bacterial cultures of blood, and synovial fluid analyses have been described in several case reports of arthritis in sea turtles (Harms et al. 2002, Greer et al. 2003, Raidal et al. 2006, Innis et al. 2014, Innis & Frasca 2017, Powell et al. 2021). However, to our knowledge, there are no published descriptions of the cumulative results of the abovementioned diagnostic methods in healthy sea turtles, particularly when previous studies have shown that bacteremia could appear in clinically healthy reptiles (Hanel et al. 1999, Innis et al. 2010, Waugh et al. 2017). Therefore, the aim of the present study was to describe the characteristics of synovial fluid in clinically healthy sea turtles and provide a baseline value for turtles with septic arthritis.

2. MATERIALS AND METHODS

2.1. Animals and basic examination

The rescued green turtles were recruited between September 2019 and June 2022. The green turtles were either stranded, rescued from floating in the sea, or were fishery bycatch, and were subsequently registered in the sea turtle rescue system under the authority of the Ocean Conservation Administration, Ocean Affairs Council, and the Executive Yuan of Taiwan. After rescue, the individuals were transported to the rescue center of the National Taiwan Ocean University, National Museum of Marine Biology and Aquarium, or Penghu Fisheries Research Institute, based on the discovery location. Sampling was performed at the National Taiwan University Veterinary

Hospital or Rehabilitation Center. Basic information and examinations, including species, estimated age, reasons for rescue, rehabilitation time, body weight, morphometric measurements, results of physical examination, and overall radiographic examination, were obtained. The subjects enrolled in this study met the following criteria: normal attitude and appetite, normal motor function of the 4 limbs, no signs of joint swelling, and no ongoing use of antibiotics for at least 1 mo. Animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of National Taiwan University (IACUC Approval No: NTU-109-EL-00112).

2.2. Radiographic examination

Radiographs were taken for an initial assessment of the overall condition of the sea turtles to ensure that there were no radiographic joint lesions. Sea turtles from the National Taiwan Ocean University, National Museum of Marine Biology and Aquarium, and Penghu Fisheries Research Institute were examined using KXO-32S (Toshiba), Porta 120 HF (Radincon), and CMP 200 (CPI) radiographic systems, respectively. Dorsoventral vertical beam images and horizontal radiographic beams were obtained in the right lateral and craniocaudal views. Further images focusing on the bilateral forelimbs were included to examine the intactness of all joints.

2.3. Blood collection

Blood collection and culture were performed according to guidelines for human and small-animal medicine, as well as some reported blood culture studies in reptiles (Work et al. 2003, Innis et al. 2010, Towns et al. 2010, Lamy et al. 2016, De Plato et al. 2019, Wellehan & Divers 2019). Venipuncture for blood culture was performed twice at both the right and left dorsal cervical sinuses to rule out false-positive results due to potential surface contaminants or laboratory errors. The sampling intervals at these 2 sites were at least 30 min apart. For gross decontamination, the skin around the sampling sites was cleaned by alternating circular scrubs 3–5 times, each using 5% chlorhexidine and water. Final decontamination was performed using 0.5% chlorhexidine in a 70% alcohol solution (Jolife Skin Antiseptic Solution, Everstar Medical Group) using a back-and-forth scrubbing technique for 30 s under strict aseptic conditions, including the use of sterile gloves and surgical caps. The venipuncture sites were allowed to dry for approximately 30–60 s, and skin swabs (Creative Life Science) were used to detect skin contamination. Next, venipuncture was performed using a 10 ml syringe with a 22-gauge needle (Terumo Medical). The amount of blood collected was calculated in advance to avoid exceeding 3 ml kg^{-1} in order to remain within the suggested maximum volume of 8 ml kg^{-1} (Martinez-Jimenez & Hernandez-Divers 2007, Stokes & Epperly 2008). Blood collected from each side was transferred into 2 separate blood culture bottles: 1–3 ml for the aerobic bottle (Becton Dickinson) and 3–5 ml for the anaerobic culture bottle (Becton Dickinson). Each needle was replaced with a new sterile needle, and the injection port of the blood bottle was sterilized with alcohol before the blood was transferred. The remaining sample (minimum 0.8 ml) was kept for blood smear and placed in lithium heparin tubes (Vacuette®, Greiner Bio-One) for complete blood count and biochemical analysis.

2.4. Arthrocentesis

The shoulder joints were selected for synovial fluid aspiration in the present study based on the number of cases, with reference to previous reports (Harms et al. 2002, Greer et al. 2003, Raidal et al. 2006, Solano et al. 2008, Guthrie et al. 2010, Innis et al. 2014, Cruciani et al. 2019, Powell et al. 2021). Synovial fluid from bilateral shoulder joints was sampled from each individual. Before sampling, lidocaine (2%) (Tai Yu Chemical & Pharmaceutical) 2 mg kg^{-1} diluted with sterile saline was infiltrated subcutaneously around the shoulder joints for local anesthesia (Park et al. 2009, Chatigny et al. 2017, Norton et al. 2017). The puncture site was disinfected using the aforementioned procedure as an aseptic standard for blood cultures. Skin swabs were collected for bacterial culture. Under manual restraint, arthrocentesis was performed using a 1 or 3 ml syringe with a 24-gauge, 2.5 cm needle (Terumo Medical). The forelimb was extended and flexed ventrally to expose the joint space maximally. The needle was inserted dorsally at approximately 45° into the space between the humeral head and glenoid fossa (Fig. 1). The blind technique was used because the ultrasound probe would limit the sampling space and interfere with the angle of needle insertion. The procedure was repeated twice in each joint; one drop of sample was used for qualitative analysis and the rest of the sample was transferred into a new pediatric blood culture bottle (Becton Dickinson) for synovial fluid bacterial culture separated from the previous bottle for blood culture.

Fig. 1. Arthrocentesis of the shoulder joint in a green turtle. The turtle's head is to the left side of the image and the left forelimb is under manual restraint; arthrocentesis was performed using a 1 ml syringe with a 24-gauge, 2.5 cm needle (Terumo Medical). The left forelimb was extended and flexed ventrally to maximally expose the joint space. The needle was inserted dorsally at approximately 45° into the space between the humeral head and glenoid fossa, which was palpated and marked by the left middle finger of the veterinarian (H. P. Su) in the image

All procedures were performed by the same veterinarian (H. P. Su). A graphic flow chart of the sampling procedure is provided in Fig. 2.

2.5. Sample processing

2.5.1. Blood examination

Packed cell volume was calculated using capillary tubes (DIN ISO 12772 Microhaematocrit Capillary Tubes, Assistant®) after the blood was centrifuged at 14 820 × *g* for 3 min (Hsiangtai Machinery Industry). Erythrocyte and leukocyte counts were determined using the Natt and Herrick method, as previously described (Heatley & Russell 2019). All blood smears were stained using Wright-Giemsa stain (Tonyar Biotech) and were used to calculate the percentage of each type of leukocyte and the absolute counts of heterophils, lymphocytes, monocytes, basophils, and eosinophils under an Olympus CX22 LED Upright Microscope at 1000× magnification with oil immersion.

Plasma was isolated for biochemical examinations after blood centrifugation. The examined parameters included total protein, glucose, uric acid, aspartate aminotransaminase, creatine kinase, blood urea nitrogen, calcium, phosphorus, sodium, chloride, and potassium levels (Ortho Clinical Vitros®350 Chemistry System, Johnson & Johnson; i-STAT®, Abbott Laboratories). Plasma protein electrophoresis was performed at the Taipei Union Clinical Laboratory using the agarose gel electrophoresis method, as described by Gicking et al. (2004).

2.5.2. Synovial fluid analysis

Synovial fluid analysis was performed by the same veterinarian (H. P. Su) to evaluate the volume, color, turbidity, viscosity, and cytology. After aspirating the synovial fluid samples, the volume, color, turbidity, and viscosity were recorded. For turbidity, descriptors 'clear/transparent' (absence of flocculent debris) and

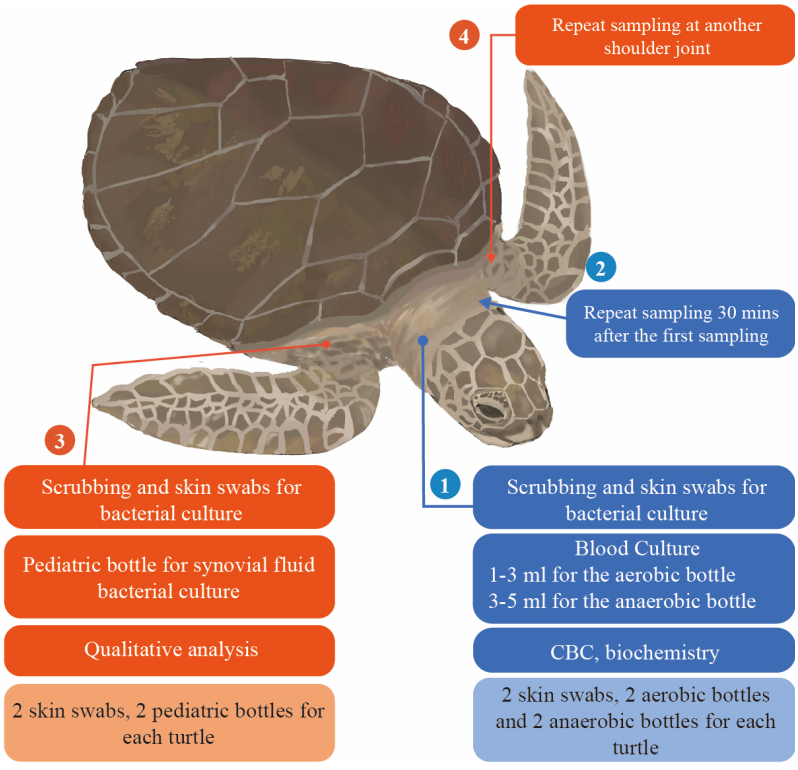

Fig. 2. Sampling procedure to clarify the site of sampling and number of bacterial culture samples of each turtle. CBC: complete blood count. Picture drawn by Jia-Chian Li

'turbid' (presence of cells, fibrin, bacteria, or crystals) were used subjectively (MacWilliams & Friedrichs 2003, Fernandes. 2013). In terms of color, descriptors 'colorless,' 'very light yellow,' 'red-tinged,' 'yellow–orange discoloration,' and 'white,' were employed subjectively (MacWilliams & Friedrichs 2003, Fernandes. 2013). Viscosity was evaluated using the tip of a needle to pull a strand from the slide on which the expressed synovial fluid was placed, and the length of the strand was determined using an electronic caliper with an accuracy of 0.1 cm, which was pre-set at 2.5 cm and placed beside the slide. Strand length was recorded as below or above 2.5 cm based on the mammalian standard (Fernandes 2013). One drop of synovial fluid was subsequently smeared for cytology, which was stained with Liu's stain (modified Romanowsky stain) (ASK® Liu's Stain A and B, Tonyar Biotech). Cells were identified under 1000× magnification by oil immersion (Olympus CX22LED), and 100 cells were counted to obtain the differential cell count. Cytological evaluation of the synovial fluid involves calculating the percentages of mononuclear cells and heterophils. Mononuclear cells include lymphocytes, histiocytes, and synovial lining cells. Given the limited clinical significance of the diagnosis and treatment of these cells (Fernandes 2013) and the fact that it is difficult to differentiate synovial lining cells from histiocytes unless they present in clusters, they were grouped together and classified as histiocytes and synovial lining cells in this study.

2.5.3. Bacterial culture

All specimens for microbial culture were transported to the diagnostic institute within 4 h of collection. To minimize transportation time, the closest laboratory was selected for different sampling sites. Samples obtained from the National Taiwan Ocean University and Penghu were sent to the laboratory at the National Taiwan University Animal Hospital or Taipei Union Clinical Laboratory, and samples collected from the National Museum of Marine Biology and Aquarium were sent to the Kaohsiung Lezen Reference Laboratory. Bacterial cultures of the blood, synovial fluid, and skin swabs were performed by certified veterinary clinical scientists or medical laboratory scientists in human medicine. Blood culture bottles were incubated in a BD BACTEC™ FX blood culture system (Becton Dickinson) for 5–7 d at 37°C (Work et al. 2003, Innis & Frasca 2017). If no growth was observed after 7 d, the blood culture was reported to be negative. If a mismatched result was obtained on different culture sets, additional samples were acquired for recheck to provide further data. The blood culture was reported to be negative if the rechecked cultures were negative. Swabs were inoculated onto blood agar plates (BAP)/eosin methylene blue (EMB) and chocolate agar plates (CAP) and incubated for 48 h at 37°C. If growth was noted, the samples from aerobic bottles were plated on BAP/EMB agar plates (Creative Life Science) and CAP (Creative Life Science), while samples from anaerobic bottles were plated on CDC anaerobic agar plates (Creative Life Science). BAP/EMB agar plates and CAP were aerobically incubated at 35°C with 5% carbon dioxide, while CDC agar plates were incubated anaerobically at 37°C. Standard microbiological techniques, such as Gram staining and culture characteristics, were employed. Additionally, commercial identification systems, the BD Phoenix M50 system (Becton Dickinson) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, were used for more precise identification. Antibiotic susceptibility testing was conducted using the automated BD Phoenix M50 system.

2.6. Statistical analysis

All data were analyzed using SAS® 9.4 Foundation (SAS Institute). Morphometric measurements, including body weight, straight carapace length, straight carapace width, curved carapace length, and curved carapace width, were recorded as mean, median, and minimum-to-maximum range. Differential cell counts of the synovial fluid were recorded as a percentage with a 90% confidence interval as well as the mean, median, and minimum-to-maximum values.

3. RESULTS

3.1. Animals

Fourteen green sea turtles were recruited between September 2019 and June 2022, of which 5 were from the National Taiwan Ocean University, 8 were from the National Museum of Marine Biology and Aquarium, and 1 was from the Penghu Fisheries Research Institute. All turtles were classified as juveniles according to their carapace length except for 1 subadult (Witherington 2017). The reasons for rescue or the major problems faced by these individuals were bycatch (n = 10), fibropapillomatosis (n = 2), fishhook ingestion (n =1), being stranded (n = 2), buoy-

Table 1. Morphometric, signalment, and rehabilitation data from 14 green turtles enrolled in this study. All sea turtles were confirmed with no evidence of joint disease or other significant systemic disorder. Est.: estimated; NE: not evaluated; SCL: straight carapace length; SCW: straight carapace width; CCL: curved carapace length; CCW: curved carapace width; NTOU: National Taiwan Ocean University; NMMBA: National Museum of Marine Biology and Aquarium; PFRI: Penghu Fisheries Research Institute

Case	Rehabilitation center	Est. age	Body weight (kg)	SCL (cm)	SCW (cm)	CCL (cm)	CCW (cm)	Rehabilitation time (until sampling)	Reasons for rescue
1	NTOU	Juvenile	12.00	NE	NE	NE	NE	1 yr 7 mo	Buoyancy disorder
2	NMMBA	Juvenile	12.51	48.2	39.7	50.5	45.3	8 mo	Stranded
3	NMMBA	Juvenile	22.33	54.6	45.2	56.8	51.8	2 yr 3 mo	Bycatch, fibropapillomatosis
4	NMMBA	Juvenile	23.32	54.8	45.4	58.1	51.0	3 yr 2 mo	Bycatch, fibropapillomatosis
5	NMMBA	Subadult	28.75	62.8	48.9	66.0	59.0	2 mo	Stranded
6	NTOU	Juvenile	10.10	41.0	38.5	44.5	44.0	12 d	Bycatch
7	NTOU	Juvenile	12.28	44.2	36.2	45.2	42.8	7 d	Bycatch
8	NTOU	Juvenile	16.44	47.6	41.3	49.0	47.3	13 d	Bycatch
9	NMMBA	Juvenile	10.10	43.4	37.0	45.3	43.9	4 mo	Bycatch
10	NTOU	Juvenile	8.45	41.5	36.3	43.0	41.0	1 d	Stranded
11	NMMBA	Juvenile	6.86	39.2	32.4	40.9	36.9	1.5 mo	Bycatch
12	NMMBA	Juvenile	10.04	40.9	34.4	43.0	39.9	3.5 mo	Bycatch
13	NMMBA	Juvenile	8.03	39.4	32.4	40.7	37.5	4.5 mo	Bycatch, fishhook ingestion
14	PFRI	Juvenile	8.92	42.0	35.0	44.6	39.5	7 d	Bycatch

ancy disorder (n = 1), and fishing net entanglement (n = 1). The mean rehabilitation time until the sampling date was 235 d (median, 82.5; range, 1–1155 d). All turtles exhibited normal activity, appetite, urination, and defecation at the time of sampling. Physical examination revealed no specific findings in any of the individuals at the time of sampling. (The 2 turtles diagnosed with fibropapillomatosis had previously undergone surgical excision before sampling.) Detailed information on the individual turtles is presented in Table 1. Of the 14 sea turtles, 7 had a history of antibiotic use (Table 2). These 7 turtles had their antibiotic withdrawal at an average of 5 mo (median, 3.5; range, 1–14 mo). Radiographic findings revealed that all joints were intact, without osteolytic lesions, at the time of sampling.

3.2. Hematologic and plasma biochemical analysis

Reference ranges for green turtle hematologic and plasma biochemical data have been described in several previous reports (Osborne et al. 2010, Suarez-Yana et al. 2016). Values outside the reference range are listed in Table 2. The abnormalities were mostly mild to moderate. No evidence of systemic infection was found in any of the subjects according to the leukocyte count, morphology, and percentage of heterophils. As none of the sea turtles had clinical signs of illness around the time of sampling, the observed modest clinicopathologic abnormalities were considered to be the result of individual variation, subclinical conditions, or

Table 2. Medical history and clinical examination findings in 14 green turtles recruited for synovial fluid analysis in Taiwan. NA: not applicable; NAD: no abnormality detected; CK: creatine kinase

Case	Antibiotic	Withdrawal period before sampling	Blood exam
1	Ceftazidime	1 yr 2 mo	Hyperglycemia (313 mg dl^{-1}), Hyperphosphatemia (12.7 mg dl^{-1})
2	None	NA	Hypoalbuminemia (0.82 mg dl^{-1})
3	None	NA	Hypercalcemia (13.4 mg dl^{-1})
4	Doxycycline	11 mo	NAD
5	Amikacin	2 mo	Hypercalcemia (12.7 mg dl^{-1})
	Enrofloxacin	2 mo	
	Doxycycline	1.5 mo	
6	None	NA	NAD
7	Enrofloxacin	1 mo	Hypokalemia (2.39 mmol l^{-1}), Hypochloremia (96.7 mmol l^{-1})
8	None	NA	NAD
9	None	NA	NAD
10	None	NA	Elevated CK (2000 U l^{-1})
11	Amoxicillin	1 mo	NAD
12	Amoxicillin	3.5 mo	NAD
13	Ceftiofur	5 mo	NAD
14	None	NA	Hyperphosphatemia (12.2 mg dl^{-1}), hyperchloremia (131 mmol l^{-1})

handling (e.g. hyperglycemia), and were not deemed clinically important (Table 2).

3.3. Bacterial culture of blood and synovial fluid

All skin swabs from either the blood sampling site or joints revealed negative culture results. Blood culture from most of the rescued sea turtles (13/14) revealed negative results at 37°C. Positive culture results were obtained from 1 sample. In Case 12, an unidentified Gram-positive bacillus from the left dorsal cervical sinus was found growing in the anaerobic bottle, whereas no bacterial growth was found in the right dorsal cervical sinus. Because of the inconsistency in the results, the blood culture was re-examined in this individual 1 mo after the first sampling. The second culture showed no bacterial growth; thus, this case was considered culture-negative. Culture results from all synovial fluid samples were negative.

3.4. Characteristics and cytology of synovial fluid

The amount of synovial fluid aspirated from the shoulder joints ranged from 1 drop to a maximum of 1 ml. Seventeen out of 28 joint aspirations yielded less than 0.1 ml, and only 2 aspirations yielded 1 ml of synovial fluid (Table 3). The synovial fluid appeared clear, transparent, and light yellow. Blood-tinged samples were considered to have been aspirated from tissue trauma caused by needle entry (Fig. 3). All synovial fluid samples were able to create

Table 3. Collection volume and differential cell counts of synovial fluid from 14 rescued green turtles in Taiwan. The extent of blood contamination is defined as +, 1–2 erythrocytes in every high-power field under immersion objective; ++, 3–4 erythrocytes; +++, >4 erythrocytes. QNS: quantity insufficient; sample volume or cellularity too low for differential cell counts

Case	Shoulder joint	Collection volume (ml)	Histiocytes and synovial lining cells (%)	Lymphocytes (%)	Heterophils (%)	Blood contamination
1	Right	0.2	62	38	0	
	Left	0.2	50	50	0	++
2	Right	<0.1	94	6	0	
	Left	<0.1	QNS	QNS	QNS	
3	Right	<0.1	QNS	QNS	QNS	+++
	Left	<0.1	QNS	QNS	QNS	
4	Right	0.6	53	44	3	++
	Left	1	85	15	0	+
5	Right	<0.1	QNS	QNS	QNS	+++
	Left	<0.1	92	7	1	+
6	Right	<0.1	QNS	QNS	QNS	
	Left	1	53	46	1	
7	Right	<0.1	68	32	0	+
	Left	0.5	QNS	QNS	QNS	
8	Right	<0.1	QNS	QNS	QNS	
	Left	<0.1	QNS	QNS	QNS	
9	Right	<0.1	QNS	QNS	QNS	+++
	Left	<0.1	60	40	0	+++
10	Right	<0.1	53	45	2	
	Left	<0.1	QNS	QNS	QNS	
11	Right	0.2	81	17	2	+++
	Left	<0.1	81	19	0	+++
12	Right	0.5	90	10	0	+
	Left	<0.1	51	46	3	+
13	Right	0.5	QNS	QNS	QNS	+
	Left	<0.1	QNS	QNS	QNS	+
14	Right	0.1	81	19	0	++
	Left	0.3	52	48	0	++

a strand of over 2.5 cm by withdrawing a needle from a droplet on a slide.

Under cytologic examination, cells were randomly distributed on slides with a pink granular proteinaceous background (Fig. 4A). 'Windrowing,' a characteristic linear pattern, was occasionally observed in the samples (Fig. 4B). Among the differential cell counts in the synovial fluid, the dominant cells were histiocytes, synovial lining cells, and lymphocytes, with a few heterophils (Fig.5). The percentages of histiocytes and synovial lining cells, lymphocytes, and heterophils at 90% confidence intervals were 61–76% (mean: 69%, median: 65%, range: 50–94%), 23–37% (mean: 30%, median: 35%, range: 6–50%), and 0–1% (mean: 1%, median: 0%, range: 0–3%), respectively (Table 3). Blood contamination was noted in 22 of 28 synovial fluid samples. Synovial lining cells (Fig. 5), characterized by large round nuclei and prominent cytoplasm, showed an oval to spindle-like appearance and were embedded in a pink matrix; they were occasionally noted and often presented in clusters on the slide.

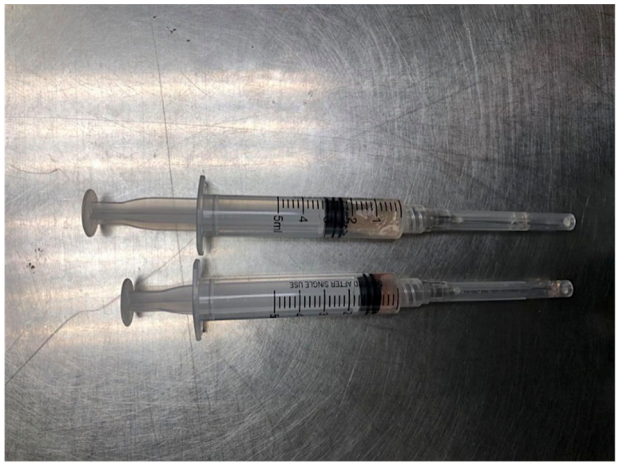

Fig. 3. Synovial fluid of green turtle aspirated from the bilateral joint. The upper sample is clear/transparent, with a very light yellow appearance, and the lower sample is blood-tinged and shows a light red color

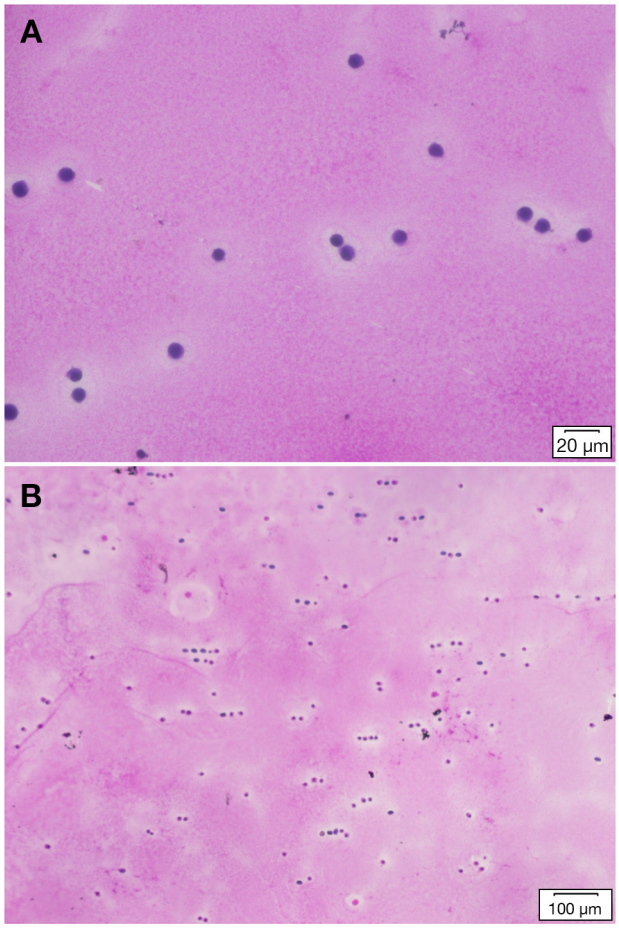

Fig. 4. Cells from the synovial fluid of a green turtle (Liu's stain). (A) Cells in normal synovial fluid presented a random distribution with a pink granular proteinaceous background. (B) 'Windrowing' distribution of cells was occasionally observed in synovial fluid samples

4. DISCUSSION

In the present study, 14 green turtles without radiographic joint lesions or evidence of systemic infection were recruited for blood cultures and synovial fluid analyses. With 1 blood culture considered false positive, bacterial cultures of both the blood and synovial fluid samples were negative at 37°C. The characteristics of normal synovial fluid in green sea turtles are generally similar to those in mammals, with a clear/transparent, colorless to very light yellow color, and a >2.5 cm strand of viscosity (MacWilliams & Friedrichs 2003, Fernandes 2013). Histiocytes, synovial lining cells, and lymphocytes dominated the cell composition, with a percentage range of 97–100%. A few heterophils were found, which generally constituted less than 3% of the normal synovial fluid. The current report describes the method of arthrocentesis as well as the qualitative analysis and cytology of normal synovial fluid in sea turtles, providing a reference for diagnostic tools. The present study supports prior recommendations that both blood culture and synovial fluid analysis can be performed simultaneously to preliminarily diagnose septic arthritis in sea turtles.

4.1. Blood culture

Previous reports on snakes, lizards, and leatherback turtles indicated that positive blood cultures commonly

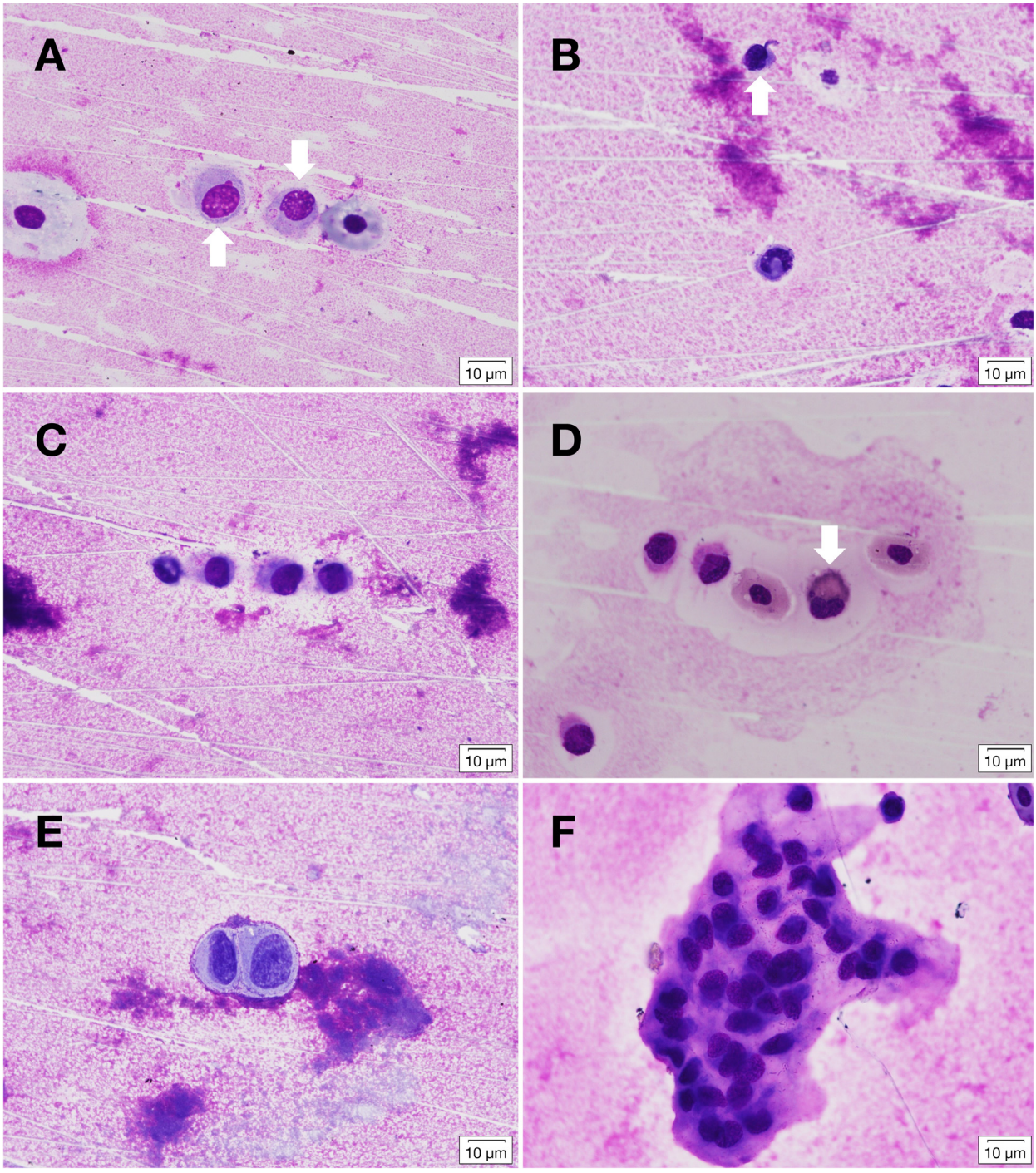

Fig. 5. Various cell types in the synovial fluid samples of the studied green turtles (Liu's stain). (A) Two histiocytes (arrows) between 2 erythrocytes. (B) Lymphocyte (arrow) next to red blood cells and mononuclear cell with U-shaped nucleus in lower fields. (C) Two histiocytes between 2 lymphocytes. (D) Heterophil (arrow) between 2 erythrocytes and 3 histiocytes to the left. (E,F) Doublet (E) and cluster (F) of synovial lining cells characterized by large round nuclei and prominent cytoplasm. Pink matrix in all photos is proteinaceous synovial fluid

exist in clinically healthy individuals (Hanel et al. 1999, Innis et al. 2010, Waugh et al. 2017). This finding may be related to the fact that the spleen of reptiles is not as efficient as that of mammals for pathogen clearing; therefore, bacteria are more commonly found in the bloodstream of reptiles (Wellehan & Divers 2019). In

green turtles, bacteremia is associated with the severity of fibropapillomatosis; approximately 86% of stranded turtles with tumors were positive for bacteria, whereas none of 48 free-ranging sea turtles with a tumor score of 0 or 1 was positive for bacteremia (Work et al. 2003). Our study matched the above findings; Cases 3 and 4 were positive for fibropapilloma with a tumor score of 1, and none had bacteremia. In contrast, our study showed that blood cultures yielded mostly negative results in the healthy rescued sea turtles. However, this should be interpreted with caution as there are some limitations to our procedure. An adequate blood volume is important for detecting bloodstream infections. A larger sampling volume may have contributed to the higher yields (Lamy et al. 2016). The recommended maximal collection volume for sea turtles ranges from 0.3 to 0.8% of their body weight in grams (Martinez-Jimenez & Hernandez-Divers 2007, Stokes & Epperly 2008). In this study, we opted for the lowest recommended collection volume for safety and utilized pediatric blood bottles, which are more suitable for sea turtles than the common bottles designed for adults. In our study, bacteria were cultured only at a temperature of 37°C. Because the average body temperature of sea turtles is approximately 25–33°C (Stamper et al. 2017), bacteria that grow optimally within this temperature range may not be cultivated. However, traditional culture temperatures, such as 37°C, are still routinely used in some laboratories currently due to the lack of a culture procedure specifically for sea turtles (Innis & Frasca 2017). A study of osteomyelitis in Kemp's ridley sea turtles used 37 and 25°C for blood culture, but the positive rate was not reported in the results section of that paper (Powell et al. 2021). Further research is required to determine the effects of temperature on sea turtle bacterial cultures.

One green turtle was found to be initially culture-positive. Negative culture results were obtained from the contralateral blood specimens. Because negative culture results were obtained from the other blood culture sets, contamination was considered most likely. In human medicine, multiple bottle sets obtained from anatomically different sites can help differentiate contamination from true bacteremia, and collecting 2 to 3 sets is generally recommended (Lee et al. 2007, Tenderenda et al. 2022). Contamination mostly appears in a single bottle of a set, whereas true bloodstream infections are usually positive in several samples (Tenderenda et al. 2022). Other factors that can be used to differentiate contamination from bacteremia include the identity of the bacteria, time to growth, quantity of growth, clinical and laboratory data, source of sampling, and automated classification using information technology (Hall & Lyman 2006). In human medicine, contamination can be identified using the species of cultivated bacteria (Hall & Lyman 2006). Given that research on sea turtle blood culture is still in its infancy, it is difficult to directly identify bacteria as contaminants or real pathogens. Sufficient examination data are required to establish a bank of pathogenic organisms found in sea turtles to serve as predictors of contamination. Possible contributors to false-positive blood cultures include high leukocyte counts, overfilled bottles, and errors in incubation (Lamy et al. 2016). Because leukocytosis and overfilled samples were ruled out in this case, an error in the culture procedure could be inferred. Although culture swabs showed no evidence of skin contamination in the individual, contamination from the skinfolds could not be entirely excluded because it was sometimes difficult to maintain neck extension throughout the procedure.

4.2. Arthrocentesis and synovial fluid analysis

Arthrocentesis can be performed in all sea turtles under local anesthesia using 2 mg ml^{-1} diluted lidocaine, which infiltrates subcutaneously around the shoulder joints. Because only mild muscle contractions were observed during the manipulation, minimal to moderate restraint was sufficient. Sedation or general anesthesia was considered unnecessary. This could be because most sea turtles in this study were juveniles and relatively calm. It would be beneficial to perform this procedure under mild sedation in larger or agitated individuals.

In mammals, cellularity can be evaluated subjectively using cell counts on slides. A normal specimen contains approximately 2 cells per field at 400× magnification (Fernandes 2013). Because the cells were not evenly scattered on the slides in our samples due to the normal viscosity of the synovial fluid, this estimation was considered imprecise. Hyaluronidase can be added to decrease the viscosity and improve cell distribution in the synovial fluid. Otherwise, cellularity should be objectively measured using total nucleated cell counts (TNCCs). TNCCs are commonly used to differentiate inflammatory and noninflammatory arthropathies in mammals (Fernandes 2013). However, the limited amount of synovial fluid obtained in our study, presence of nucleated erythrocytes, and differences in the morphology of white blood cells from mammals precluded the use of TNCCs in this study. Nevertheless, manual calculation of TNCCs of synovial fluid in sea turtles could still be a potential research topic in the future and may

be a sensitive diagnostic reference for the identification of infectious arthritis.

Histiocytes and synovial lining cells and lymphocytes were the dominant cell types among all samples in the present study; however, the percentage of these 2 cell types varied among sea turtles and among the different joints in an individual. It is sometimes difficult to differentiate lymphocytes from other non-vacuolated mononuclear cells. In small animal research, a wide reference range of lymphocytes from 0–100% to 3–28% and large mononuclear cells from 64–97% to 60–92% have been reported (Fernandes 2013). Lymphocytes are sometimes classified as mononuclear cells, and their percentages have been reported together (Fernandes 2013). Among the groups of mononuclear cells, cell type identification is of little practical importance in clinical diagnosis and therapy (Fernandes 2013). Heterophils are occasionally found in the synovial fluid and represent the dominant inflammatory leukocytes in the blood of reptiles (Heatley & Russell 2019). The presence of over 5–12% neutrophils or vacuolated phagocytically active mononuclear cells in the synovial fluid is considered abnormal in mammals (Fernandes 2013). In the present study, the percentage of heterophils was generally less than 5%, and no vacuolated or phagocytic cells were observed in any of the samples. Meanwhile, heterophilic and histiocytic inflammation were evident in fine-needle aspirate samples from affected joints (Powell et al. 2021).

Blood contamination was common because a blinded sampling technique was used in this study. Prior to this study, ultrasound-guided arthrocentesis was attempted using carcasses. As the probe limits the sampling space and interferes with the angle of needle insertion, we used a blinded technique by identifying the anatomical landmark of the humerus rather than using echo-guided aspiration. This approach may be more practical for sub-adult or adult turtles with larger anatomical structures. Advanced image-assisted techniques such as fluoroscopy or computed tomography (CT) guidance, which have been applied in human medicine, can also be used to target the synovial cavity (Hansford & Stacy 2012). In our experience, extending the forelimb away from the body and slightly pressing it down can maximally expose the space in the shoulder joint and easily locate the humerus. However, this approach is somewhat difficult to apply to larger turtles, such as subadults and adults, or to individuals with good body condition and heavy soft tissue coverage. The use of a spinal needle with either ultrasound, fluoroscopic, or CT guidance or direct surgical biopsy is warranted in these animals.

4.3. Limitations and future work

Given the strict inclusion criteria, our sample size was small. Samples for bacterial culture were sent to different laboratories, which could have introduced bias. However, a complete investigation protocol was applied to all selected individuals, and the samples obtained provided substantial information that could provide basic data on the various diagnostic tools for septic arthritis in sea turtles. Further research on bacterial culture at the ambient temperature experienced by sea turtles, as well as comparisons of the diagnostic results in affected sea turtles, could be conducted to complement the diagnostic information, and this may potentially help identify risk factors for septic arthritis in sea turtles.

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