Bull. Nippon Vet. Life Sci. Univ., No.67, 64-64, 2018.

Studies on coronaviruses causing enteric infections in domestic animals in Japan

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(Conferred on 21 September 2017, VA-183)

Coronaviruses cause a wide range of diseases in farm and domestic animals, some of which are a threat to the farming industry and give serious issue on the economy. In 2013, a huge porcine epidemic diarrhea (PED) outbreak has occurred in Japan after a period of 7 years of absence, which causes high morbidity and mortality in piglets. On the other hand, bovine torovirus (BToV) causes mild to moderate diarrhea in claves. BToV isolated from diarrheal specimens has the full length hemagglutinin-esterase (HE) gene; however, the viruses lose the HE protein as a result of mutation of the HE gene following several passages in cultured cells. In the present study, phylogenetic and antigenic characterization of newly isolated porcine epidemic diarrhea virus (PEDV) in Japan is described. Moreover, biological activity of the HE protein of BToV has been explored.

To evaluate the mechanism by which a large outbreak of PED occurred in Japan, where the majority of sows are vaccinated, we isolated two new strains of PEDV from the intestines of piglets and found that they showed greater similarity to US isolates (group II PEDV) than to the Japanese vaccine strain (group I PEDV). We compared the antigenicity of the vaccine type strain and newly isolated strains by means of neutralization test using sera from a number of pigs from various farms and showed that they are antigenically similar. This is the first report of the similarity of group I and II viruses using sera from individual pigs vaccinated with group I virus. These data suggest that the large outbreak of PED in Japan may not be attributed to inefficient vaccination but may be due to the extremely high virulence of the newly appearing viruses.

BToV, which causes diarrhea in calves, contains the HE protein on the viral envelope when isolated from the host, although HE is often lost from the virion after multiple

passages in cultured cells. This suggests that HE protein may be important for replication or pathogenesis in infected animals, but is not indispensable for the replication in cultured cells. In the present study, we explored the biological functions of the HE protein. We isolated the BToV Niigata-3 (Nig-3) from diarrheal specimen of the cattle and cloned Nig- 3-3 with HE (HE+) and Nig-3-8 without HE (HE-) using human rectal tumor (HRT-18) cells and compared their growth in cultured cells. Nig-3-8 (HE-) grew more efficiently than Nig-3-3 (HE+), suggesting the possibility that HE inhibits viral growth in culture. It was found that the interferon (IFN)- α reduced the infection of HE- virus in cells, but not that of HE+ viruses, whereas IFN- β had no influence on their growth. HE protein expressed in human embryonic kidney 293T (HEK 293T) cells were examined whether HE works as IFN- α antagonist by using Sindbis virus. The infection of Sindbis virus in HEK 293T cells expressing HE was not affected by the IFN- α treatment, though the infection was depressed in cells expressing the truncated HE after treatment of IFN- α . This indicated that HE protein acts as an IFN- α antagonist. These results collectively suggest that HE plays an important role in the pathogenesis in BToV infections as an IFN antagonist to innate immunity.

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Bull. Nippon Vet. Life Sci. Univ., No.67, 65-65, 2018.

Treatment Strategy Studies in Canine and Feline Refractory Epilepsy

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(Conferred on 8 March 2018, VA-184)

Epilepsy is one of the most common brain disorders and is encountered most frequently in humans, cats, and dogs. In veterinary medicine, epilepsy is almost exclusively treated with antiepileptic drugs (AEDs) therapy, while surgery has yet to be performed in dogs and cats with drug-resistant epilepsy. Additionally, the operative procedure and complication of epilepsy surgery have not been reported in veterinary medicine. Therefore, the surgical indication criteria and methodology to detect the epileptogenic zone (EZ) are essential to be explored in veterinary medicine. The purpose of this study was to develop epilepsy surgery as a new treatment for dogs and cats with refractory epilepsy. Therefore, the risk factors for survival in dogs and cats with epilepsy were investigated in the referral hospital in Japan to create indications for epilepsy surgery, and advanced magnetic resonance imaging (MRI) techniques were evaluated to detect the EZ using an animal model of mesial temporal lobe epilepsy (MTLE). Additionally, anterior temporal lobectomy (ATL), which is a common epilepsy surgery in humans with MTLE, was performed in healthy dogs in order to assess the surgical procedure and complications. This retrospective study proposed the indication criteria for epilepsy surgery in dogs as "cases with high-frequency epileptic seizures (≥ 0.3 seizures/month) who do not respond to applicable AEDs and/or who have a detectable EZ (i.e., focal epilepsy)." Additionally, the present study showed that the voxel-based morphometry and diffusion and perfusion MRIs were useful for detecting the EZ. Proton Magnetic Resonance Spectroscopy showed the ability to evaluate the EZ laterality and indicated functional changes in the epileptic brain following treatment with zonisamide, which is a commonly used AED. Among the seven healthy dogs who were subjected to ATL, five (71%) had a successful resection of the hippocampus and amygdala, while the remaining two were euthanized during the operation due to uncontrollable hemorrhage from the middle cerebral artery. The most common surgery complications were atrophy of the ipsilateral temporal muscle and absent or decreased contralateral menace response. However, following successful ATLs without detectable iatrogenic injury on postoperative MRI, there were no complications. The results of the present study suggested that ATL may be an applicable technique in dogs with MTLE. Although further studies are still needed in order to investigate the efficacy of ATL in dogs and cats with MTLE and improve the surgical precision, we assume that epilepsy surgery can be established in veterinary medicine. In addition, epilepsy surgery for companion animals with epilepsy will allow histopathological evaluations of resected tissues, which will contribute to our understanding of epileptogenesis.

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Bull. Nippon Vet. Life Sci. Univ., No.67, 66-66, 2018.

New evaluation markers for energy metabolism in various species

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(Conferred on 27 April 2017, VB-325)

Enzymes, involved in the metabolism of glucose and fatty acids and in energy homeostasis, are crucial in maintaining life, and may be variable among animal species, tissues, and various physiological and nutritional status of a given animal. In particular, elevations in MDH activity and MDH/LDH (M/L) ratio likely indicate heightened energy metabolism, as MDH of the malate-aspartate shuttle, contributes to the coupling of glycolysis with ATP production via the transport of cytosolic NADH into mitochondria. The malate-aspartate shuttle, one of the NADH shuttles, transports cytosolic NADH, a key molecule for oxidative metabolism and ATP production, into mitochondria, and plays an important role in the regulation of mitochondrial metabolism in animal cells.

Enzyme activities of energy metabolism, such as MDH, M/L ratio, glutamate dehydrogenase (GLDH), and fructokinase (FK) of the feline leukocytes were compared against those of the canine leukocytes. Significantly lower MDH, M/L ratio, GLDH, and higher FK, pyruvate kinase, and G6PD may reflect the unique demands and usages of nutrients and energy sources in cats with higher incidence of obesity, insulin resistance, and diabetes mellitus compared to dogs. Elevations in cytosolic and mitochondrial MDH and the resultant cytosolic M/L ratio are noted in cells that may be experiencing increased mitochondrial ATP productions. Elevated ATP productions, in turn, provide for increased energy demands (intense exercise, neoplastic cell growth, acute weight gain). Type 1 diabetic dogs with higher plasma glucose concentrations, showed lower levels MDH, M/L ratio, as well as AST, as compared to the healthy control dogs. These reflected the defect in glucose usage and uptake as energy source in the peripheral tissues, resulting in increased circulating plasma glucose concentrations seen in diabetic patients. Decreased activity levels of MA shuttle enzymes may be one of the characteristics of energy metabolism in diabetic dogs, and may be useful as a diagnostic indicator to monitor the overall metabolic condition of the diabetic patients, identify those at risk, and predict the treatment outcome. Racehorses demonstrated higher plasma MDH and LDH activities, and

significantly higher plasma M/L ratio as compared to those of the riding horses. Moreover, racehorses demonstrated significantly higher levels of lipid metabolites as compared to those seen in riding horses. Racehorses, under intense training, may have adapted to the demands of higher activity levels by increasing muscle mitochondrial respiration, oxidative capacity, and fat utilization of the skeletal muscles as energy source in order to process and consume energy more efficiently. In experimentally overfed dogs with acute weight gain, significant elevations were noted in lipid metabolites, glucose, leukocytic MDH and LDH. Although not significant, plasma and leukocytic M/L ratios showed mild increase in the over-fed group after the feeding trial. In conclusion, M/L ratio in plasma and leukocytes reflected positive energy balance and increase in energy metabolism associated with overfeeding.

A better grasp of trends in shifts of the energy metabolism enzymes may help understand the species-species differences in energy production and usage, and early detection and prevention of energy metabolism dysregulations such as diabetes, neoplasia, and obesity. Further studies on various disease models and metabolic states in different species may assist in a better understanding of their clinical usage.

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Bull. Nippon Vet. Life Sci. Univ., No.67, 67-67, 2018.

Studies on development of new diagnostic system with laparoscopy for obesity in dogs and cats

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(Conferred on 21 September 2017, VB-326)

Feline obesity was defined as "Primary Obesity" and "Secondary Obesity". The primary obesity was then divided into those with or without health issues, and the former was termed pathological obesity (obesity disease), the later was termed simple obesity. Pathological obesity (obesity disease) was further divided into subcutaneous fat obesity and visceral fat obesity or metabolic syndrome. We studied the efficacy of laparoscopic surgery as a tool for diagnosing metabolic diseases. Previous reports have suggested that the release of adipokines from adipose tissues decreases in laparoscopic surgery compared to an open surgery owing to its low level of surgical stress.

Because the levels of MDA, INS, ADN, and COR were lower in dogs that underwent laparoscopic ovariohysterectomy compared with dogs that had an open surgery, it was suggested that laparoscopic surgery enables the observation of abdominal organs such as the liver and kidney possibly effected by metabolic disease under minimum surgical stress.

By performing a laparoscopic liver biopsy on cats with liver lipidosis and conforming that the M/L ratio was low in such cases, which suggested that the ATP generating ability of feline liver cell with adipose degeneration deteriorates. We performed a laparoscopic surgery and a CT scan in cats with a BCS of 3, 4, or 5, which enabled a direct observation of the state of aberrant peritoneal fat accumulation as well as a biopsy of adipose and liver tissue. As BCS increased, the level of blood serum ADN decreased, while the increase in peritoneal fat accumulation was evident. Seeing that cats with BCS 4 already had aberrant fat accumulation on the liver and other intraperitoneal organs, as well as decreased blood serum ADN level, it was suggested that even in cats with BCS 4, the small fat cells would start to evolve into enlarged fat cells which release insulin resistance hormone. When managing obesity, it is necessary to build a treatment strategy based upon an accurate evaluation of the current body weight status. This would require development of a biochemical marker and a quantitative scaling system for each corresponding stage of the disease. The metabolome markers deemed appropriate were malate dehydrogenase / lactate dehydrogenase activation ratio which reflects energy metabolism, HDL / LDL ratio reflecting the lipid metabolism, and triglyceride concentration. In addition, the change in blood concentration levels of insulin and adiponectin are also indispensable as a diagnostic marker. Blood concentrations of ALT and AST are useful indication for changes in liver enzymes. High-sensitivity CRP, TNF-α, MCP-1, and interleukin are inflammatory marker, whereas MDA concentration as lipid peroxide marker, SOD and GSHpx acitivities as antioxidant enzyme marker are also effective for diagnosing obesity. By performing more of the same procedure and increasing the number of data, we aim to establish the obesity diagnostic criteria in dogs and cats.

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Bull. Nippon Vet. Life Sci. Univ., No.67, 68-68, 2018.

Studies on Paracoccidioidomycosis ceti

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(Conferred on 22 February 2018, VB-327)

In this study, I report about the new knowledge about the paracoccidioidomycosis ceti (PCM-C) in cetaceans in Japan regarded as a candidate of zoonotic disease, and I represent this new information to keep healthy for Japanese people as marine nation

In chapter Ⅱ , the first report of PCM-C in Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and similar symptom case that is also in the same kinds of dolphin are describe. It includes the detail of clinical symptoms of the dolphins , examinations, progress and molecular biological considerations. In addition, I also suggest that *Paracoccidioides brasiliensis sensu stricto* could be a causative agent of PCM-C. From these findings, it is likely that the Japanese coastal waters have already contaminated with PCM-C.

In chapter Ⅲ , I describe about the epidemiological survey of PCM-C using immunohistochemistry examination with sera obtained from some cetacean species kept in Japanese aquaria, that aim to establish one of the method to diagnose PCM-C. As a result, 61.0% of the samples show positive reaction against PCM-C antigen. From the feature, it is possible to suggest that considerable numbers of the reared small cetaceans in Japan could possess anti-PCM-C antibody.

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Bull. Nippon Vet. Life Sci. Univ., No.67, 69-69, 2018.

Effect of glucagon like peptide-1 receptor agonists on gastrointestinal transit in healthy dogs

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(Conferred on 8 March 2018, VNT A-9)

Glucagon like peptide-1 (GLP-1) receptor agonists have various pharmacological activities, thereby which ameliorate glycemic control in patients with diabetes. Especially, delay of gastrointestinal transit rate is important for decreasing postprandial hyperglycemia. However, few studies have reported the effect of GLP-1 receptor agonists on gastrointestinal transit time (gastric emptying time and small intestine transit time) in healthy dogs. In this study, we firstly examined whether the APAP method (evaluation of gastric emptying) and the SASP method (evaluation of small bowel movement) can use for evaluating gastrointestinal motility as in dogs. Furthermore, final objective of this study was to evaluate the influence of GLP-1 receptor agonists on canine gastric emptying time and small intestine transit time using the APAP and SASP method.

We evaluated the validity of commercial APAP detection kit, measuring canine serum levels after the administration of APAP. Serum APAP concentration below 5 μg/ml were not able to evaluate accurately. Next, we evaluated the accuracy and reproducibility of serum APAP and SP concentration using HPLC in dogs. It would be better to measure the serum APAP concentration within 7 days. For investigating canine gastrointestinal motility, the APAP and SASP method and the Barium impregnated polyethylene spheres (BIPS) were compared. BIPS could not use for evaluating gastrointestinal motility in dogs. Next, the APAP and SASP method and the liquid contrast medium on canine gastrointestinal motility were compared. Additionally, blood metabolites were also compared. We considered that the APAP and SASP method is a reliable to evaluate the rate of gastric emptying time and small intestine transit time without photographing X-ray. Finally, we investigated the effect of GLP-1 receptor agonists (Exenatide and Liraglutide) on the gastrointestinal transit time and blood metabolic marker using the APAP and SASP method in healthy dogs. As a result, GLP-1 receptor agonists delayed gastrointestinal transit time. Furthermore, delayed gastrointestinal transit time induced lowering postprandial blood metabolic marker.

In conclusion, we established a simultaneous assay method using high performance liquid chromatography method of serum APAP and SP concentration in dogs. Additionally, we confirmed that the APAP and SASP method is a reliable to evaluate the gastrointestinal transit time. Furthermore, we found that the GLP-1 receptor agonists delayed gastrointestinal transit time in healthy dogs. These results indicate that the APAP and SASP method can use for monitoring the gastrointestinal motility in dogs and GLP-1 receptor agonists may be useful for glycemic control in diabetes dogs in the future.

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Bull. Nippon Vet. Life Sci. Univ., No.67, 70-70, 2018.

Studies of the Control of *Salmonella* Contamination in Oil Meal and Oil Meal Production Environments

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(Conferred on 20 July 2017, VNT B-1)

Salmonella contamination has long been a problem for o il meal, which is used as a vegetable protein source in compound animal feed. Although the *Salmonella* contamination rate in oil meal has been declining in recent years, cases of contamination continue to arise sporadically. Gi ven that the *Salmonella* contamination rate of oil meal production environments remains high, outbreaks of oil meal contamination are likely to be the result of secondary contamination from prod uction facilities . Therefore, in this study, in addition to evaluating *Salmonella* detection methods suited for oil meal manufacturing plants , we investigated methods to control *Salmonella* in the actual production process and in processing environments with the goal of reducing *Salmonella* contamination in finished oil meal and oil meal production environments .

First, we evaluated the delayed secondary enrichment (DSE) method, which relies on efficient isolation for detection of *Salmonella* in oil meal production environments . The DSE method exhibited high detection sensitivity in samples such as the base materials for oil meal, which contain low numbers of bacterial contaminants. In addition, in many samples, the DSE method was able to detect *Salmonella* strains with O-antigen that could not be detected using the official method for detecting Salmonella in animal feed . The above results demonstrate that the DSE method is an effective detection method for epidemiological investigation s in feed manufacturing facilities.

Next, we assessed the state of *Salmonella* contamination and e valuated methods for controlling *Salmonella* contamination in production environments. A comparison of disinfectants used to clean factory floors revealed surfactants whose main active ingredient is polyoxyethylene alkylether to be superior in terms of disinfection efficacy, safety, and cost performance. We also found that the contamination rate could be substantially reduced by combining the three measure s of disinfecting the soles of shoes, applying smooth floor coatings, and disinfecting factory floors.

Further, we assessed the state of *Salmonella* contamination and evaluated methods for controlling contamination in oil meal production lines. *Salmonella* was detected in areas of the production line that tend to accumulate large amounts of residue , suggesting that *Salmonella* survives for extended periods of time in residue. Accordingly, the contamination rate of oil meal was significantly reduced ($p < 0.05$) by removing and discarding residue from the production line after the heating stage and removing and discarding fines, which were found to have high contamination rates, from the process rather than adding them to the meal as is done conventionally.

Finally, we assessed a rapid, easy method for detecting *Salmonella* that

would be suitable for outgoing freight inspections. We were able to detect *Salmonella* in as little as 8.5 h by combining MP medium with QuickGene-mini80 and QUALIBAX™ system.

In this study, detection and control metho ds were evaluated in actual oil meal production plants and production lines. As such, they can be applied in many animal feed and food -production plants that handl e powders .

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Bull. Nippon Vet. Life Sci. Univ., No.67, 71-72, 2018.

Analyses of molecular structure and kinetics of myokine FGF21 in dogs.

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(Conferred on 8 March 2018, VNT-55)

Myokine is a general term for physiologically active factors secreted from skeletal muscles and affect metabolism. Myokines secreted in response to exercise stimulations have various effects on many kinds of organs such as adipose tissues and liver via blood circulation. Although many molecules have already been reported as myokines, this study focuses on FGF21, one of fibroblast growth factors (FGFs). FGF21 is a secretory type protein consisting of 209 amino acids in humans. In addition to skeletal muscle, it is expressed throughout the body, much especially in the liver, pancreas and white adipose tissues. In skeletal muscles stimulated with FGF21, glucose uptake is promoted, calorific value is increased, insulin sensitivity is enhanced, metabolism is promoted, and anti-obesity effect is exerted. Furthermore, it is demonstrated that blood FGF21 concentration could be used as a clinical biomarker in some diseases. If canine FGF21 has the same effect with that in human beings, it would be possible that FGF21 is effective for diagnosis and treatment of some metabolic disorders, such as liver disease and obesity. However, there are few reports on FGF21 in veterinary sciences, and currently available data are extremely limited. In this study, first we cloned canine FGF21, clarified the molecular structure and tissue distributions. Next, we analyzed the blood concentrations of FGF21 in healthy dogs and determined the normal values. Finally, we investigated the influence of exercise and various kinds of diseases such as inflammation, tumor and liver diseases.

1. Canine FGF21; cDNA cloning and analyses of the molecular structure

We cloned FGF21 cDNA from canine skeletal muscle. We designed primers referring to predict sequences of canine FGF21 in the DNA database and carried out RT-PCR. In the results, canine FGF21 was composed of 209 amino acids and showed 84 to 90% identities to other animals such as cats, cattle, and humans. We investigated mRNA expressions of canine FGF21, and it was expressed in most organs, such as colon, liver, pancreas, kidney, spleen, hypothalamus, spinal cord, bone marrow, fat, skeletal muscle and skin. We also investigated gene expressions of canine FGF21 in incubated canine tumor cell lines, such as COS-C, CMS-C and CMS-J. FGF21 was expressed in any cell line, and gene mutation was not observed in all strains. These cell lines would be available for in vitro studies of canine FGF21 in futures.

2. Validation of canine FGF21 assay and blood concentrations in healthy dogs.

We have confirmed the validity of ELISA assay kit for human FGF21 using polyclonal antibodies in dogs and measured blood FGF21 concentrations in healthy dogs. In the results, reproducibility, dilution linearity and recovery were fine, and it was concluded that this kit appeared to be useful for the measurement of canine FGF21. Measured values in 15 healthy dogs, the concentration of FGF21 in the blood was 0.21 ng/mL on average, 1.1 ng/mL at the maximum, 0.011 ng/mL at the minimum, and 0.051 ng/mL at the median. there was no significant difference between male and female, young and aged dogs. It was impossible to determine the reference value because of the small sample size, but the values were approximately similar to those in human study. Additionally, there was no diurnal or daily

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variations. Fasting and feeding did not cause any effect on blood FGF21 in dogs.

3. FGF21 in dogs after treadmill-exercise and with clinical disorders

We investigated the influence of exercise and effects of various diseases on blood FGF21 concentrations in dogs. Three beagle dogs were exercised on a treadmill for 10 minutes at a maximum speed of 7 km/h every day for 2 weeks (including interruptions for 2 days). In the results, blood FGF 21 concentration tended to increase in week 1 in a dog, and decreased in week 2 in the other dog, however we did not find any clear rules. It was concluded that higher intense exercise should be required to detect any significant change of FGF21 in dogs.

Next, we measured FGF21 concentrations of 38 obese dogs, and the results were as follows, average 0.23, minimum 0.085, maximum 0.84 and median 0.198 ng/mL. Compared to the healthy group, the obese group showed significantly higher values than healthy dogs, but the difference in the values was very small, and we concluded that obesity has significant, but little effect on canine FGF21.

Finally, we measured blood FGF21 concentrations in dogs with various diseases. In the results, the median value of FGF21 in 33 dogs was 0.538, the minimum value was 0 (under the detection level), the maximum value was 1.981, and the median was 0.338 ng/mL, indicating much higher values than healthy dogs. In the next analysis, the data of 4 dogs were removed because they lacked detailed information, and we have classified other 29 dogs to inflammation, tumor and liver disease categories in order to compare FGF21 changes in different types of disorders. In the results of the tumor group, the average value was 0.627, the minimum value was 0.037, the maximum value was 1.981 and the median value was 0.361 ng/mL. In the results of inflammation group, the mean value was 0.29, the minimum value was 0.21, the maximum value was 0.404 and the median value was 0.27 ng/mL. In the results of liver disease group, the average value was 0.603, the minimum value was 0.135, the maximum value was 0.404 and the median value was 0.519 ng/mL. FGF21 concentrations in tumor, inflammation and liver disease groups were significantly higher than that in the healthy group. Based on the findings, FGF21 should be a new blood marker for tumor and/or liver diseases. By accumulating more information about the relationship between FGF21 values and their malignancy and/or prognosis, FGF21 will be available as a unique blood marker for tumors and liver diseases of dogs in animal hospitals.

4. Conclusion

Based on the above results, we have established basic information of canine FGF21. FGF21 is expressed in many canine organs. The molecular structure is similar to those of other species including human beings, suggesting that canine FGF21 has similar functions also in dogs. We can measure blood FGF21 concentrations in dogs using a human ELISA kit (however, the data will be relative values). The average blood concentration of FGF21 in healthy dogs was 0.21 ng/mL (0.011-1.1 ng/mL). There was no gender or age difference. There was no diurnal or daily variations. Fasting and feeding did not cause any effect on blood FGF21 in dogs. Mild exercise did not change blood FGF21 concentrations in dogs. FGF21 showed slightly but significantly higher values in obese dogs. In dogs with tumor, inflammation and liver diseases showed significantly higher FGF21 values than healthy dogs (much higher in dogs with tumor and liver diseases). FGF21 might be a new blood test item which reflects tumor and liver diseases, and metabolic condition in dogs.

Bull. Nippon Vet. Life Sci. Univ., No.67, 73-74, 2018.

Investigation on intraoperative body temperature lowering inducing factor and monitoring method in dogs

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(Conferred on 8 March 2018, VNT-56)

Perioperative hypothermia is a common complication in human and canine surgical patients. In human and dog, homeostatic derangements in thermoregulation from anesthesia and abnormal vasoconstriction can produce alterations in cardiac, renal, hepatic, and hemostatic functions. Further, perioperative hypothermia can cause various adverse outcomes; for example, a slower recovery from anesthesia and a longer time to extubation in dogs. Therefore, veterinary nurses responsible for anesthetic assistance during surgery should consider countermeasures to prevent intraoperative hypothermia. However, there are only few reports about the perioperative body temperature changing of the dog, the hypothermic risk factors in dog are not clarified. Furthermore, despite the significant importance of accurate temperature measurement for clinical management of dogs, the specific method for monitoring of correct core temperature is not shown. In this study, we investigated the risk factor of intraoperative hypothermia and the monitoring method of the core body temperature in dogs.

In the first chapter, to investigate intraoperative body temperature fluctuation and risk factors of hypothermia during surgery in dog, 110 anesthesia records of canine clinical patients, that received abdominal or thoracic surgery under general anesthesia at Animal Medical Center of Nippon Veterinary Life Science University (NVLU-AMC) during April 2016 to March 2017, were reviewed retrospectively. The records had detailed description about the body temperature during introduction of anesthesia to extubation were included in this study, and those with insufficient description were excluded. In abdominal surgery group, core body temperature fluctuations were compared by age, body surface area /body weight ratio (BSA/BW ratio), coat length, time of total anesthesia, respectively. To investigate hypothermia on extubation time, we compared extubation time by severity of postoperative hypothermia. We also examined differences in core body temperature fluctuation due to abdominal and thoracic surgery. As a result, in the abdominal surgery, significant decreasing of core body temperature were observed in elderly dogs and small dogs with high BSA/BW ratio. Therefore, clinical patients with such factors must be considered to have a risk of severe intraoperative hypothermia. Also, there was significant decreasing of core body temperature in long hair coat dogs, against expectations. However, the number of short hair dogs were fewer than long hair dogs used in this study, it was suggested that clinical studies involving a larger number of dogs are required to confirm this finding. In comparison by anesthesia time, as the total anesthesia time prolonged, hypothermia became severe and increasing incidence of severe postoperative hypothermia(<36.0 ℃). Therefore, if prolongation of anesthesia time is expected in abdominal surgery, it may be necessary to actively warm up during surgery and it must be considered about individually optimal warming method. Extubation time of hypothermic cases was longer than normal body temperature cases. There was no difference in body temperature fluctuation between abdominal and thoracic surgery. However, measurement site of body temperature (esophagus or rectum) cannot specify in this retrospective study. In general, it is regarded that a body temperature may vary depending on the measurement sites related with surgery type (abdominal/thoracic surgery). Therefore, we next evaluate prospectively that the differences on measurement value of each sites in abdominal and

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thoracic surgery.

In the second chapter, to evaluate the differences in body temperature fluctuation at esophagus and rectum after incision in abdominal and thoracic surgery. This study included 8 dogs of abdominal surgery and 4 dogs of thoracic surgery, all clinical cases were received abdominal/thoracic surgery at surgical oncology unit of NVLU-AMC. As a result, both esophageal and rectal temperature significantly decreased in abdominal surgery than thoracic surgery. It might be caused that exposure of visceral organ surface to relatively cool outside air were larger in abdominal surgery than in thoracic surgery. In postoperative body temperature of thoracic surgery, it was found that more cases were classified as moderate and severe hypothermia in esophageal temperature. This phenomenon thought to be caused by measurement site. Therefore, to establish a more appropriate monitoring method for core body temperature, we compared the difference between esophageal and the rectal temperature in abdominal and thoracic surgery. As a result, the esophageal temperature of thoracic surgery was significantly lower than the rectal temperature. It suggested that measurement value of esophageal temperature was descended by probe exposed by outside air when opening the chest cavity in thoracic surgery. Therefore, as a more accurate monitoring method of body temperature, it is considered that rectal temperature measurement is recommended because the esophageal temperature may show low value in thoracic surgery. On the other hand, no significant difference was found between esophageal temperature and rectal temperature in abdominal surgery. However, most cases were surgery in upper abdominal incision in this study. Therefore, at least in the upper abdominal surgery, it became cleared that there was no effect on the measured value by selecting either esophageal temperature or rectal temperature.

In conclusion, there was cleared risk factors of hypothermia in canine abdominal surgery. Furthermore, this study revealed appropriate measurement site of body temperature during abdominal and thoracic surgery. The veterinary nurses responsible for anesthesia assistance at perioperative period should be considered individually optimal countermeasures preventing for intraoperative hypothermia according to these results.

Bull. Nippon Vet. Life Sci. Univ., No.67, 75-76, 2018.

Investigation of positive and adverse effects of radiotherapy for canine brain tumor

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(Conferred on 8 March 2018, VNT-57)

Radiotherapy is commonly used for treating tumors in human and veterinary medicine. Radiotherapy is often used as sole therapy. Radiotherapy is also used with chemotherapy and/or surgical therapy. The therapeutic objective is relief of clinical signs, it is often performed for improving quality of life of animals. This therapy is less invasive without pain and a topical therapy, so there are lower systemic adverse effects than chemotherapy. However, adverse effects due to the radiation influence on normal tissues and tumor may occur. Radiotherapy is applied in veterinary medicine as tumor is difficult to removed by surgery because of site of occurrence. Adverse effects of radiotherapy on brain tumor occur in the brain. In order to evaluate the adverse effects, image inspection such as CT or MRI under anesthesia is required. As nurses are involved in radiotherapy in human medical care, veterinary nurses also need to be involved in radiotherapy. Therefore, veterinary nurses need to know about radiotherapy and the effect and adverse effect of radiotherapy for each disease. In human medicine, there are textbooks and research reports on nursing care of radiotherapy. However, in veterinary medicine, there are few reports that veterinary nurses involve radiotherapy. Therefore, the purpose of this study was to investigate the effect and the adverse effects of radiotherapy for canine brain tumor.

In the first chapter, we investigated the effect and adverse effects of radiotherapy against pituitary tumors in dogs. We retrospectively investigated the clinical signs and blood examinations and MRI findings after radiotherapy. Radiotherapy for canine pituitary tumor was effective for reduction of tumor size and improvement of neurologic signs. However, the relatively high recurrence of neurologic signs was observed. Its recurrence is thought to be related to the occurrence of intratumoral hemorrhage by MRI findings. In MRI findings, otitis media was also recognized, and accompanying hearing loss was observed. RT did not induce any significant changes in the dogs' basal plasma ACTH concentration and pre- and post-ACTH serum cortisol concentrations.

In chapter 2, we investigated the effect and adverse effects of the combined use of radiotherapy and hydroxyurea on canine meningioma. We retrospectively investigated abnormal findings of neurologic signs and MRI findings after radiotherapy. There is no report on this combination therapy in canine meningioma. From the results, the combined use of radiotherapy and hydroxyurea was effective for reducing tumor size and improving neurologic signs. However, the relatively high recurrence of neurologic signs was observed. Its recurrence is thought to be related to the occurrence of intratumoral hemorrhage by MRI findings. The incidence of otitis media was low, but it may be caused by the radiation field. In addition, it was revealed that the combined use of radiotherapy and hydroxyurea lengthened the survival time as compared to the past reports. From the above, it was suggested that the combined use of radiotherapy and hydroxyurea improve neurologic signs, reduce tumor size, and possibly prolong survival time.

In this study, we were able to present informed consent of radiotherapy for canine brain tumors. Veterinary nurses did an important research to know the knowledge, effect and adverse effects of radiotherapy. In addition, regular follow-up including

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after radiotherapy was considered to be important for early detection of abnormal findings due to radiotherapy MRI examinations. In the future, as a prospective study, it is necessary to give information of radiotherapy for owners.

Bull. Nippon Vet. Life Sci. Univ., No.67, 77-78, 2018.

Study of a Sustainable Activity Carried Out by NPO and Volunteers those Work for Reduction of the Number of Cats be Culled.

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(Conferred on 8 March 2018, VNT-58)

In Japan, The ownerless cats are the most numerous of the animal be culled by Healthcare Center. This paper focuses on 'Coping with ownerless cats' and 'protection and adoption of the cats'. I studied the sustainable activity carried out by NPO and volunteers.

In chapter 1, I first investigated issues of Action Plan, which is published by Ministry of the Environment. The Action Plan aims happy symbiosis of people and animals. In this Plan, NPO and volunteer are expected as a key player. Second, I studied issues of NPO and volunteer in other studies. Other studies have concluded that NPO and volunteers have some problems.

For example, they have difficulty with working as a volunteer. Because the volunteer works for no pay, they need to have another work to earn money. Consequently, they find it difficult to be successful at both work and volunteer work.

Based on the above, this study reveals that the Action Plan needs some kind of support system for volunteers and NPO.

In chapter 2, I studied point of sustainable activities and issues in three cases of volunteers and NPO: First of all, activity carried out by me myself as an individual volunteer with neighborhood association; secondly, the activity

of volunteer organization whose name was MUSASHIONO community cats organization (volunteer organization); and at last, the activity of NPO whose name was Tokyo Cat Guardian (TCG).

In the first case, the individual volunteer attempted to build a good relationship with other volunteer organizations and a neighborhood association before starting volunteer work. Volunteer organizations helped an individual volunteer with technical, financial and public relations of volunteers work. I discovered that in the case of an individual volunteer good communication with the people and activity in small area would make the activity sustainable.

However, there were some problems that an individual volunteer couldn't solve troubles between residents and people who were feeding ownerless cats. In addition, if board members of neighborhood association are changed, the new members might disagree to this subsidization scheme.

In the second case, volunteer organization, working together MUSASHIONO city government, gained resident's confidence, and support and was making use of SNS to get many supports.

On the other side, there were some issues for them. First, the burden that TrapNeuterReleace (TNR) for ownerless cats increased by people lives in another city. This was because of SNS. Secondly, the volunteer organization had to depend on donations. Thirdly, this current system makes heavy demands on a representative of voluntary organization.

In the third case, I studied to the points of sustainable work and issues of work by TCG. They gained money as social business. Furthermore, they employed stuff, surveyed the needs of people and cat. On the other side, there were some issues for them. As they expand business, they were looking for stuff. However the personal expenses hold the first place in the expenditures. In addition, they lacked of business model for cats those were transferred to new owners.

In chapter 3, I suggested that ideal measures, support systems and socioeconomic infrastructure

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investment for volunteers and NPO. Overviewing the cases, it became clear that the individual volunteer, volunteer organization and NPO were faced with some problems when they promote activities. For this reason, I investigated the model and social maintenance for sustainable activities. As an individual volunteer, it is desirable for the individual volunteer to deal with familiar problems. In addition, it is necessary that the individual volunteer cooperate with volunteer organizations and neighborhoods association.

If works be complicated by consultants from residents, an individual volunteer needs to corporate with local governments.

As a volunteer organization, they can share their volunteer works with members, corporate with local governments, focus on public, for getting support from surrounding people. In addition, a volunteer organization needs to cooperate with other volunteer organizations for covering issues of volunteer activities. On the other side, university, company and animal hospital need to corporate with them as much as possible. That will lead to image improvements of organization. I thought it would be better to corporate as benefiting both of the supporter and the recipient.

As NPO, they can take the seminar of management and participate in the gathering for getting stable income. Furthermore, they always have to pay more attention to needs of human and animal. It may lead to earn personal expenses and maintain their business.

In addition, it is necessary to make the income and expenditures clear so that they would not to be taken as driving benefits from animal.

Finally, all of the cases those I dealt with in this study could not but depend on contributions. It is inevitable to develop social environments those make them easy to use crowdfunding. It is necessary to change the social recognition that people who would like to get a cat would choose animal protection society as a first choice from where to adopt one.

Bull. Nippon Vet. Life Sci. Univ., No.67, 79-80, 2018.

Analysis of reproductive status and genetic connectivity among local populations of feral raccoon (*Procyon lotor*) in Miura Peninsula, Kanagawa Prefecture

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(Conferred on 8 March 2018, VNT-59)

The raccoon (*Procyon lotor)* was introduced to Japan from North America. Kanagawa Prefecture has been conducting the Kanagawa Raccoon Control Program to protect the native fauna and flora. In Miura Peninsula of Kanagawa Prefecture, the feral raccoon population density has been evaluated with the relative index based on Catch per Unit Effort (CPUE). The index suggested that population density was decreasing due to the Kanagawa Raccoon Control Program. However, it was probably not enough to state the effect of the program in recent years. Thus, we hypothesized that may reduce the effect of control of the raccoon population with three next factors;

(1) High population growth in all part of Miura Peninsula
(2) Number of immigrated individuals from other local populations exceeding that of captured raccoons
(3) Deficiency of the catch efforts.

In this paper, we assessed reproductive status, a sex-age structure, connectivity among populations and CPUE with the aim of clarify the effects of these three factors.

We used individuals caught in Miura Peninsula, Kanagawa Prefecture from February 2016 to March 2017, and in Kamakura City from April 2005 to March 2007.

In Chapter 2, We analyzed reproductive rates, including pregnancy rates , litter size, and mortality rates by sex-age structure, that were varied population growth, as aimed to clarify if the factor (1) has been occurred. These data were compared between Miura Peninsula and Kamakura City. The pregnancy rate of 1-year-old (34.5%) females were significantly lower than that of ≧ 2-year-old (92.5%) in Miura Peninsula. In comparison to Kamakura City (1-year-old : 64.9% , ≧ 2-year-old : 78.1 %), the pregnancy rate of ≧ 2-year-old in Miura Peninsula were not found significantly different, but 1-year-old were significantly row. In Miura Peninsula, the mean number of litter sizes were not significantly different between 1-year-old (mean 3.7) and ≧ 2-year-old (mean 3.9). In addition, comparing the mean number of litter sizes for respective ages between ones in Miura Peninsula and in Kamakura City (1-year-old : mean 3.7, ≧ 2-year-old: mean 4.0) were not found a significant difference. Therefore, we suggested that the reproductive rates may have been decreased. Because the sex-age structure was not significantly different between in Miura Peninsula and in Kamakura City, we considered that a variation of the mortality rates which may give a large impact on the population densities have not been caused. However, the sex-age structure did not change, nevertheless the reproductive rates were decreased. Considering these facts, decreasing mortality rates seemed to compensate for decreasing sizes of the birth population by decreasing the reproductive rates may have been happened on the population. For the reasons above, we suggest that factor (1) is less likely to occur.

In Chapter 3, we defined the local populations in Miura Peninsula with analysis of population genetics. Also, we indirectly assessed a migration of individuals among the local populations with analysis of genetic differentiations, as aimed to clarify if the factor (2) has been occurred.

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As a result of analysis of population genetics, the feral raccoon population in Miura Peninsula was constructed with five clusters. Moreover, we defined the five local populations (Zushi, Hayama, Yokosuka-West, Yokosuka-East and Miura) for convenience, considering distribution of the clusters. Miura was showed to be low genetic connectivity with other local populations, because F_{ST} and R_{ST}, indices of genetics differentiations, indicated large differentiations. Thus, Miura was considered that an effect by a migration of individuals from other local populations was low. Among Zushi, Hayama, Yokosuka-West and Yokosuka- East, F_{ST} indicated small genetic differentiations in a part of combination, but R_{ST} did not seem genetic differentiations. Thus, we considered that the genetic connectivity among these local populations was high. For these reasons, we suggest that an effect of factor (2) has been small in Miura. However, the population sizes may have increased by a migration of individuals in other four local populations, because the genetic connectivity was high.

In Chapter 4, we analyzed the changings of catch efforts and CPUE (the number of the captch / the number of catch efforts × 100 Trap Night per 100 km2) per the local population, as aimed to clarify if the factor (3) has occurred. In Miura, which was seemed a low genetic connectivity, catch efforts have been about 400 TN (Trap Night), and CPUE has been maintained less than 0.5. In contrast, in Yokosuka- West and Yokosuka-East, which were seemed a high genetic connectivity, catch efforts have maintained about 400 TN, but CPUE has been higher than the one in Miura (more than 0.5 less than 1.0). Furthermore, in Zushi, CPUE has been barely changed less than 0.5, when the catch efforts have maintained about 1000 TN. In Hayama, catch efforts have been less than about 250 TN, and CPUE has been twice as much as other local populations. Therefore, it is likely that the high genetic connectivity regions need more catch efforts than the low genetic connectivity regions to maintain the CPUE low. In terms of the factor (3), we considered that current catch efforts have reached adequate quantities, in order to maintain CPUE to less than 1.0, that is, the areas lacked enough CPUE, the catch pressure, to accomplish further decreasing CPUE. Especially, Hayama needs higher catch efforts to reach additional decreasing of the feral raccoon population.

Thereafter, we consider that the combination of factor (2) and (3) caused decreasing of the performance of the catch effort. However, it is suggested that affects of these factors we are small between that regions with low genetic connectivity.

Bull. Nippon Vet. Life Sci. Univ., No.67, 81-82, 2018.

Behavioral traits dog owners in Japan prefer -Evaluation based on owner, dog trainer reports and behavioral observation-

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(Conferred on 8 March 2018, VNT-60)

Small breed dogs like Miniature Dachshund, Toy Poodle and Chihuahua are the most popular breeds in Japan (Japan Kennel Club 2016) while larger breeds are preferred in Europe and US. These differences suggest that owner expectation may differ between Japan and Europe or US. In order to understand what owners expect upon obtaining a dog in Japan would be useful information for welfare of the dogs and for researches as well. Since many of the questionnaires or dog personality scales available are developed in Europe or US where larger breeds are popular, it may not be the best tool for assessing dogs and owners in Japan.

In this study, in order to understand the owners preferences and satisfaction in Japan, I have 1) confirmed the validity of the Japanese version of dog personality test using web survey (Nose et al. 2017) by dog owners/trainers evaluating the same dog, 2) examined the predictive validity of the scale by measuring dog behavior directly, and 3) conducted a web survey on owner's attitudes and expectation on their dogs with using the Japanese version of the dog personality scale.

In order to conform the concurrent validity of the dog personality scale developed dog instructor-owner pairs were asked to evaluate the same dog. The original dog personality was developed by Kubinyi et al. (2009) with web survey in Germany. Nose et al (2017) conducted a web survey in Japan and developed the Japanese version with the scale consisting 19 items with four factors. Factors are "calmness", "dog sociability", "boldness", and "trainability". The concordance between the professional dog trainers and dog owners would indicate the validity of the scale. Ninety-three dog owners and 18 dog trainers participated. Dog breeds were Toy Poodle, Labrador retriever, Jack Russell Terrier, Chihuahua, Miniature Dachshund, Mix, Shiba Inu, and others. The correlational coefficients between factor scores by the trainer and the owner were as follows; Calmness ($r=0.88$), Dog sociability($r=0.73$), Boldness ($r=0.72$) and Trainability($r=0.65$) at $p<.01$ level. The results suggest that owner's evaluation is in congruent with that of the professional dog trainer, thus the validity of the scale is strong.

Thirty-one dogs participated in behavior tests focused on home situation, walking situation, travel situation and the vet clinic situation. A feeding puzzle is also presented to the dog. The result of the dog personality scale and the result of the dog behavior tests were compared to determine the predictive validity of the scale. Dog breeds were Mix, Toy Poodle, Border collie, Dalmatian, Labrador retriever and others. Owners were told they could stop the test at any time. Dogs were allowed to roam around the room prior to the tests. The session was videotaped and owners were allowed to use treats if they like to. After the test, they were asked to fill in the dog personality test.

The correlational coefficient of result of the dog personality scale and the behavior test were as follows; Calmness and the at home situation ($r=0.42$), and Trainability and the travel situation ($r=0.36$) at $p<.05$ level.

Dogs which showed interest in puzzle scored higher in Trainability than the ones with no interest ($t(29)=2.52$, $p<.05$, $d=0.94$). Dogs which solved the puzzle scored marginally higher in Dog sociality than which did not ($t(29)=1.73$, $p<.10$, $d=0.64$). Except for the Boldness,

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the predictive validity of the dog personality test was strong.

A web survey with 500 participants in large cities in Japan was conducted. The dog personality scale questions and questions related owner expectations and attitudes. After deleting incomplete or insufficient data, 370 of them were analyzed. Dog breeds were Chihuahua, Miniature Dachshund, Mix, Shiba Inu among others. The demographics of the breeds were similar to that of Japan Kennel Club data(2016). Multiple regression analysis indicate that owner satisfaction is related Calmness(β =.312) and Trainability(β =.230) and tameness of the dog is also related to Calmness(β =.297) and Trainability(β =.218).

The results of dog trainer and owner questionnaire, and the dog behavior tests confirmed the validity and the predictive validity of the scale. Even though the number of dogs participated in the behavior tests are limited, the dog personality scale is shown to be good for use in assessing dogs in Japan.

Japanese owners were satisfied with dogs with calmness and trainability. It is similar to what parents in Japan expect in their children. When asked what are the important characteristics, parents say 'good child' who does not disturb others and get along with others while American parents focused on independence (Azuma 1998). By conducting similar survey in Europe and US may lead to better understanding of what a dog is to human being.

The Japanese version of dog personality scale used here can be a good assessment tools both in application such as dog shelters, vet clinics or breeding. and in researches such as a measure to identifying certain genes related to behavior.

Acknowledgement

Nippon Veterinary and Life Science University Ethical Committee (S29H-12) approved this study.

Bull. Nippon Vet. Life Sci. Univ., No.67, 83-84, 2018.

Survey of environmental airborne bacteria and prevention of nosocomical infection in Nippon Veterinary and Life Science university teaching hospital

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(Conferred on 8 March 2018, VNT-61)

Nosocomical infection by drug-resistant bacteria, for example methicillin-resistant Staphylococcus aureus (MRSA), is a serious problem in both human and veterinary medical field. For prevention these problem, the guidelines and manuals which indicate treatment of infected patients, house keeping, and standard precaution should be observed by health care worker. In recent veterinary medical field, interest in drug-resistant bacteria or nosocomical infection is increasing. However, most of veterinary hospitals have less recognition of hygiene environment and prevention measures based from scientific evidences. Especially, veterinarian and veterinary nurses need to actively contribute the environmental improvement in their hospital.

In this study, we investigated about hygiene management status by culturing surface bacteria attached on various places in Nippon Veterinary and Life Science university (NVLU) teaching hospital and verified for prevention of nosocomical infection at hospital environment.

Chapter 1 : Investigation of microbial status in NVLU teaching hospitals.

We researched at microbiological contamination on the floor and cages in intensive care unit (ICU), canine-hospital room, and feline-hospital room in our hospital from November, 2016 to April, 2017. Surface samples of the floor (10 cm × 10 cm) was wiped using sterilized swab. The swab sample was preserved in 1 mL NaCl as a test material, and 100 μL of samples was cultured onto the bacterial medium immediately. Heart Infusion Agar (HA), DHL Agar (DHL), and Mannitol Salt Agar (MA) were used to culture general bacteria, *Enterobacteriaceae, and staphylococcus* spp., respectively. The cultured media were incubated at 37℃, 24 hour, under aerobic condition. After then, we counted bacterial colony. As the result of colony count, there were $10^2 \sim 10^3$ CFU/ 100 cm^2 of bacteria on the floor in each room. One of the most contaminated place was in front of the hospital cage. The floor of ICU had highest number of bacteria and number was $9.3 \times 10^2 \pm 360$ CFU/100 cm^2. As the Results of colony count in the cage, there was comparable contamination to each room. Especially, we observed higher number on the door, handles, and the bottom and the corner of cages. We collected 270 samples total, and observed 257 samples had bacterial growth on HA (257/270; 95.2%), 43 samples on DHL (43/270; 15.9%), and 169 samples on MA (169/270; 62.5%), respectively. This result convince that Staphylococci has been isolated with higher rate on the floor in the NVLU teaching hospital.

Chapter 2 : Identification of the Staphylococci isolated from the NVLU teaching hospital.

Since many staphylococci were isolated from chapter 1, we further analyzed these staphylococci to identify.

We analyzed 87 staphylococcal strains isolated from the floor and 21 staphylococcal strains isolated from the cage. We picked up 1 colony on HA into 200 μL distilled water for DNA extraction and heated on 100℃ for 10 minutes then centrifuged it at 4℃, 5 minutes, 12,000 rpm. The supernatant was used PCR as template DNA to identify *S. aureus, S. epidermidis, S. pseudintermedius, and S. shleiferi* using each species specific primer pair.

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Additionally, in order to detect methicillin-resistant Staphylococci, we checked mecA gene using PCR. As regards methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) isolated from samples, we had epidemiological survey to compare the origin with MRSA and MRAP isolated from inpatient which hospitalized at the same period. Staphylococci which not be identified with PCR had classified by coagulase possession as coagulase-positive staphylococci (CPS) or coagulase-negative (CNS). As the results of identification of staphylococci species, most strains were identified as coagulase-negative staphylococci (49/87) or *S. pseudintermedius* (25/87). These staphylococci has higher possession probability of mecA gene; coagulase-negative staphylococci was 32.7% or *S. pseudintermedius* was 52.0%. In addition, the results epidemiological analysis of MRSA using

Coagulase type test and POT analysis proved that environmental staphylococci and inpatient staphylococci were identical. Furthermore, the results epidemiological analysis of MRSP using RAPD showed identical analogous DNA band pattern. These result suggest that there were drug-resistant bacteria in our hospital and it possibly contaminates inpatient or the floor.

Chapter 3 : The effects of hospital infection measures on environmental contamination.

It is important that daily cleaning or disinfection tasks to keep hygiene in the hospitals. However, it has not been functioning well in our hospital. We surveyed about the bacterial number on soles wearing by veterinary nurse to figure out whether shoes would be one of the factor to contaminate the floor. There were $10^2 \sim 10^3$ CFU of bacteria on the foot. Same component bacteria such as MRSP or MRCNS were detected from the soles of shoes and floor. In addition, we installed sticky sanitary mats at entrance of the room to test whether they could effectively improve sanitation management in our hospital. Same bacterial culture methods were used as chapter 1, and we compared the colony number before and after installation of mat. There were no significant difference the number and spices of bacteria before and after of installation of mat. There were $10^2 \sim 10^3$ CFU/ 100 cm^2 of bacteria on the floor in each room. These results suggested that this mat is no effect to improve measures for environmental contamination, such as decreasing in the number of bacteria or removal of MRSP. The hospital infection measures should be considered further.

It has been often discussed that hospital infection or spreading of drug-resistant bacteria. In human medicine field, infection control team (ICT) has been organized to respond its problems and these problems should be worked together in medical team. As regards in veterinary medicine field, this study would be the alarm on current hospital environment. Moreover, we hope to our study enlighten hospital staff to improve present contamination state.

Bull. Nippon Vet. Life Sci. Univ., No.67, 85-86, 2018.

Studies on methods for measuring copy-numbers of target-genes per genome by quantitative PCR

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(Conferred on 8 March 2018, ALS-90)

【Introduction】

Our laboratory has been analyzing copy-numbers of mitochondria in eukaryotic cells, plasmids in lactic bacteria, and integrated DNA in genomes of recombinant yeasts.

Analyzing methods in our earlier studies were measuring the quantities of genomes and target DNAs by absorbance of 260nm (A260), or image-analyzing of agarose-gel stained by intercalator after electroporation and compared the DNA concentrations with standard DNAs. In the later studies, we used quantitative PCR (qPCR) to calculate quantities of the target DNA in the recombinant yeast genomes to elucidate their copy numbers.

However, those methods have following problems:

A. In order to measure concentrations of DNA by A_{260}, preparation of large quantity of DNA is necessary. It is not very easy to prepare large amount of DNA from yeast cells, especially *P. pastoris*, since they have firm cell-walls. And preparation of enough amounts of standard DNAs by PCR for measuring A_{260} is also very difficult.

B. Precise measurements of DNA quantities is also difficult with gel-image analysis, since genomic DNAs degrade during preparation from cells, and they tend to appear as smearing bands at lower molecular weights in agarose gel electrophoresis.

C. Even using low absorption tubes or chips, standard DNA curves are not linear at higher dilution of standard DNA in qPCR measurements.

Moreover, there is no standard cells whose copy-number of the target DNA have been confirmed. That was also preventing the precise analysis of copy-numbers of target-genes of our target recombinant yeast cells.

【Objects】

To solve the problems described above, and to construct a new method for precise estimation of the copy-numbers of target-genes per genome.

【Materials】

Bacteria: *Pichia pastoris* GS115 stain (purchased from Life Technology).

Escherichia coli, 5-alpha F' I^q, competent cells (purchased from New England Biolabs)

A GFP expression vector fragment (containing 3 to 9 tandem GFP expressing gene fragments) was introduced by electroporation, and strains in which GFP protein expression had been confirmed were used for DNA extraction and analysis.

【Methods】

<DNA extraction and purification methods, and confirmation of their DNA purity>

Extraction of total DNA was performed with GFP expressing stains cultured on YPD plates at 30 ℃ for 2 days using ISOPLANT DNA Extraction kit (Nippon Gene Co., Ltd.).Further DNA purification was carried out using Fast Gene Gel/ PCR Extraction kit (Japan Genetics Co., Ltd.), and purity of the DNA samples was confirmed by agarose gel electrophoresis.

<Measurement of putative DNA concentrations>

Image analysis of agarose gel electrophoresis and absorbance of 260nm (A_{260}) were used for calculations of putative DNA concentration of the samples.

<Low absorption tubes and chips>

Low absorption disposable plastics were purchased from Eppendorf Co., Ltd. (Germany).

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<Yeast tRNA>

Yeast tRNA was purchased from Sigma (USA), and used for dilution of DNA samples in 100 mg/mL solution to prevent absorption of diluted DNAs to the plastics.

<Copy number measurement using quantitative PCR >

Quantitative PCR was performed using Eco-Real-time PCR system (Illumina). The URA3 gene (one copy per genome) was used for standard genome copy-number. Copy number of the *GFP* gene of the DNA samples were measured using a GFP-and-URA3 copy-number standard DNA, which was a restriction-enzyme-digested-linearized plasmid into which one *GFP* gene and one *URA3* gene were introduced.

【Results and Discussion】

Effect of using low absorbance plastics

We observed prevention of absorption of DNAs to the plastics in some extents by using those low absorption plastic tubes and chips, and we could store DNA solutions in the concentration of ng/mL for longer periods of time. However, in the case of concentration of a few hundred pg/mL or lower, we could not obtain a linearity at the standard DNA curve with qPCR. Thus, we considered addition of some materials that could prevent the further absorption of diluted DNA in the samples. Blue dextran or herring sperm DNA are often used for this purpose. However, we supposed that those might be interfering qPCR measurements. Finaly, we chose yeast tRNA to prevent the absorption of diluted DNAs to plastic tubes and chips.

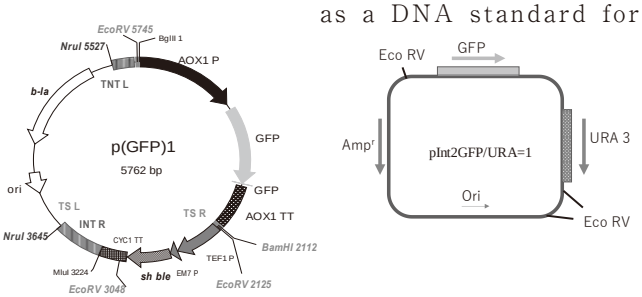
Effect of adding tRNA in diluents

Yeast (*S.cerevisiae*) tRNA (Sigma) was added at the concentration of 100 ng/mL to the diluents to prevent diluted DNAs in the samples to stick to the surface of the plastic tubes and chips. As we supposed, tRNA worked very well to prevent further absorption of diluted DNA in the samples, and we could observe a linearity of samples with DNA concentrations of pg/mL in qPCR.

Thus, using low-absorption plastics, and adding tRNA 100mg/mL in diluents, we could improve the linearities of qPCR measurements even at the DNA concentrations of pg/mL.

Construction of a vector with genome: target gene = 1:1 (pINT2GFP/URA3=1)

We considered that, if there had been standards in which their target-gene copy-numbers had already been known, it would be possible to measure the copy-numbers of target genes per cells more precisely. Hence, we constructed a new standard DNA (vector) for this purpose. *P. pastoris ura3* gene DNA was amplified from genomic DNA by PCR, and introduced into a GFP vector[1,2], p(GFP)1, that contained one copy of GFP gene. A resultant vector, pInt2GFP/URA=1, which contained one GFP gene and one URA3 gene (See Figure), was linearized with a restriction enzyme, Bam HI, and used as a DNA standard for qPCR.



Using this kind of DNA as a DNA standard, it is considered possible to obtain more accurate data on copy-numbers of target genes per genome with qPCR.

Figure.

A GFP vector, p(GFP)1, (left) and the constructed genome gene (URA3) : Introduced target gene (GFP) = 1 : 1 vector, pInt2 GFP/URA=1, (right)

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Bull. Nippon Vet. Life Sci. Univ., No.67, 87-88, 2018.

Comparative analysis of cell differentiation of bone marrow-derived mast cells between BALB/c and C57BL/6 mice.

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(Conferred on 8 March 2018, ALS-92)

【Introduction】

Allergy is a disease caused by excessive action of immune reactions against antigens (allergens) harmless to humans and animals. Representative examples include asthma, pollinosis, atopic dermatitis, food allergy and the others. By Gell and Coombs in 1963, allergy was largely classified into four types. Common allergies are classified as type I. There are differences in the likelihood of allergies in individuals, which are greatly affected by factors such as inherent genes, surrounding environment, intestinal flora and socio-psychological stress. Symptoms of allergy are initiated by activation of immune cells in the beginning.

There are various kinds of immune cells, among which T cells are mainly divided into helper T cells and killer T cells. The direction of the immune response is mainly regulated by two T cell lines, helper T cell type 1 (Th1) and helper T cell type 2 (Th2). It is known that Th1 cells primarily produce cytokines such as IFN-γ and regulates cellular immune responses, and Th2 cells primarily produce IL-4, IL-5, IL-13 and others. It has been believed that it controls the immune response. The Th1/Th2 balance in the immune response varies depending on the immunization method, the type of infection, and the mouse strain. For example, BALB/c mice are Th2 dominant strains which easily lead to antibody responses after infection and immunity, and are often used for the study of allergic reactions. On the other hand, C57BL/6 (B6) mice are Th1 dominant strains that easily lead to cellular immunity. The differences between BALB/c and B6 mice are reported not only in T cells but also in substances produced by macrophages.

Mast cells are large cells with granules and play an important role in allergic reactions such as hay fever and bronchial asthma. Mast cells are derived from bone marrow progenitor cells, and after migrating into peripheral tissues, they differentiate and mature under the influence of cytokines provided from the surrounding environment. Mast cells have a high affinity IgE receptor (FcεRI) on the cell surface, and when IgE bound to FcεRI are crosslinked with multivalent antigens, liberation of calcium ions (Ca^{2+}) stored in the endoplasmic reticulum is promoted, and in turn results in degranulation, and secretes inflammatory mediators contained within intracellular granules. Inflammatory mediators include histamine, serotonin, prostaglandin, β-hexosaminidase and so on.

It is unclear as to whether there are functional differences in mast cells between BALB/c and B6 mice, which are known for T cells as effector cells in allergic reactions.

In this study, we investigated whether the in vitro induced bone marrow-derived mast cells (BMMC) have functional differences among mouse strains. IL-3 is a hematopoietic growth factor that stimulates and proliferates myeloid progenitor cells and differentiates them into various blood cell types. We analyzed cell surface molecule expressions by staining, degranulation, and intracellular signaling molecules by western blot using BMMC differentiated with IL-3.

【Material and Method】

Bone marrow mast cells were prepared by flushing and collecting bone marrow cells from femur and tibia of BALB/c and B6 mice, and then adding 20% of culture supernatant of IL-3 producing cells (WEHI-3) to RPMI1640 and culturing for 3 weeks or more. BMMCs were stained for the cell surface marker FcεRI (PE)/c-Kit (Alexa488) or FcεRI (PE) / CD49b (FITC) / c-Kit (PerCP) and confirmed with a flow cytometer (FACS).

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In addition, BMMCs were stimulated with calcium ionophore A23187 or antigen (DNP-HSA), and the rate of degranulation was measured. In addition, western blot analysis of BMMCs was performed using antibodies against p-Tyr, p-Akt, p-ERK, ERK, and β-Actin.

【Results and Discussion】

When bone marrow cells were cultured in the presence of IL-3, a clear population of FcεRI$^+$c-Kit$^+$ mast cells appeared from Day 3 in BALB/c-derived cells and Day 7 in B6, and it seemed that the differentiation process of BALB/c-derived cells preceded transiently, but on Day 21 it became about the same rate. Approximate 35% were occupied by FcεRI$^+$CD49b$^+$ basophil-like cells in B6 cells on Day 7 to 14, but decreased as the number of days of culture increased. The differentiation process to BMMCs and/or differentiation rate was shown to be different between BALB/c and B6 mice. In the degranulation reaction for BMMCs, BALB/c is more prone to release granules by stimulations of both calcium ionophore and antigen, but the total amount of β-hexosaminidase activity is slightly higher in B6 in many experiments. That indicates that mast cells have a functional difference between BALB/c- and B6-derived cells. Moreover, the purity of BMMCs was not related to efficiency of degranulation. In western blot analysis using anti-phosphorylation antibody, the pattern of tyrosine phosphorylation before and after stimulation was different between BALB/c and B6 cells, and phosphorylation of Akt was strongly detected in BALB/c cells.

Next, we examined whether cell differentiation and maturation progressed furthermore in long-term culture. In the culture for more than 50 days, the proportion of FcεRI$^+$CD49b$^+$ cells that had remained on Day 21 was further reduced, and about 80 to 95% of cells differentiated into FcεRI$^+$c-Kit$^+$ cells. When the degranulation reaction was attempted again using these cells, BALB/c derived cells were more sensitive to stimulation constantly, and the total amount of β-hexosaminidase activity was again higher in B6 derived cells. In western blot analysis, phosphorylation of Akt and ERK was more strongly detected in BALB/c than in B6 cells. These differences in signaling events are thought to be involved in the difference in degranulation rate via FcεRI.

These findings demonstrated that functional differences exist in BMMCs depending on mouse strain. Differences in these BMMCs could also be responsible for at least a part of the differences in immune responses in mouse strains.

Bull. Nippon Vet. Life Sci. Univ., No.67, 89-90, 2018.

Light Elements Stable Isotope Analysis of Rice Aiming at Determination of Its Geographic Origin

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(Conferred on 8 March 2018, ALS-93)

Background and Objectives

Price of rice differs by its quality. For example, cultivar Koshihikari harvested in Uonuma region, Niigata Prefecture, Japan is recognized as high quality tasty rice and sold for a high price. Such kind of branded rice exists in other countries. False labeling of branded rice is a worldwide problem, and establishment of technique for geographic origin verification of rice is required. One of the technique for geographic origin determination is based on light elements stable isotope analysis, which needs geographical database of the light element stable isotope ratios.

In this study, rice samples were collected from 16 countries, and their light element stable isotope ratios were analyzed aiming at establishment of the international database for rice. This analysis showed trend of the isotope ratios of rice in the most countries. Variation of the stable isotope ratios within Japan was also investigated for rice harvested in regions from Hokkaido to Okinawa Prefecture. In addition, effect of cooking on the light element stable isotope ratios of rice was investigated.

Materials and Methods

1. Rice samples for stable isotope analysis

Rice samples with verified geographic origin were obtained from 16 participating countries of FAO/IAEA Project (RAS5062) in 2016 fiscal year. Rice samples collected from prefectures in Japan by National Food Research Institute, National Agriculture and Food Research Organization in 2006 fiscal year were also analyzed.

2. Stable isotope ratio analysis of rice.

Brown rice was polished and powdered. For analysis of stable isotope ratio of oxygen ($^{18}O/^{16}O$), the powdered rice samples were weighed in a silver capsule and decomposed at 1450 °C to CO. For analysis of stable isotope ratios of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$) and sulfur ($^{34}S/^{32}S$), the powdered rice samples were weighed in a tin capsule and combusted at 1000 °C to CO_2, N_2 and SO_2, respectively. The stable isotope ratios were analyzed using an isotope ratio mass spectrometer (IsoPrime 100, Isoprime Ltd.). Standards of oxygen, carbon, nitrogen and sulfur were Vienna Standard Mean Ocean Water (VSMOW), Vienna Pee Dee Belemnite (VPDB), Air and Canyon Diablo Troilite (CDT), respectively. The isotope ratios were expressed using conventional δ nonation (‰) relative to the standards.

3. Analysis of change in stable isotope ratios of rice by cooking

Rice harvested in Uonuma region in 2015 was cooked using water with different $\delta^{18}O$, water obtained at the foot of Mt. Fuji, tap water of Musashino City, Tokyo, Japan and deep ocean water obtained off the coast of Muroto Cape, Kochi Prefecture, Japan, of which $\delta^{18}O$ was -10.1 ‰, -7.9 ‰ and -0.5 ‰, respectively. The polished rice was washed 4 times with water and cooked in an electric rice cooker (NJ-HM10, Mitsubishi Electric Corporation). $\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$ of the polished raw rice, each washed rice and the cooked rice were measured as described above after lyophilization.

Results and Discussion

$\delta^{18}O$ value of plant samples reflects that of rain water, river water and ground water of the area where the plants were grown. Generally $\delta^{18}O$ value is low for plants grown in high latitude and high altitude area. $\delta^{13}C$ value of plants reflects isotope fractionation of

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their carbon fixation enzyme in stomata and becomes generally high in low humidity area. δ^{15}N value of plants reflects amount of organic materials in soil. Plants grown on organic-rich soil show high δ^{15}N value. δ^{34}S value also reflects δ^{34}S of soil on which the plants were grown.

1. Light element stable isotope ratios of rice harvested different countries

Japanese rice samples showed low δ^{18}O value, and the average was significantly lower than those of Australia, Bangladesh, China, Myanmar, Malaysia, Pakistan, Sri Lanka, Thailand, the United States and Vietnam. The average δ^{13}C value of Japanese rice samples was significantly higher than those of Bangladesh, Myanmar, Malaysia, Philippines, and Sri Lanka. The average δ^{15}N value of Japanese rice samples showed significant difference to those of Bangladesh, China, Myanmar, Sri Lanka, Thailand, and Vietnam while the δ^{15}N value of rice samples distributed around 4 ‰ . δ^{34}S value of Australian rice exhibited especially high. The average δ^{34}S value of Japanese rice samples showed significant difference to those of Australia, Myanmar, Philippines, and Sri Lanka.

The low δ^{18}O value and the high δ^{13}C value of Japanese rice may be due to the higher latitude and the lower humidity of rice cultivation area in Japan compared to other rice cultivation countries. When the δ^{18}O value of each sample was plotted against the δ^{13}C value, distribution area of Japanese rice was separated from those of other countries except Bangladesh, China, Taiwan, Myanmar, and India. This suggested possibility of discrimination of Japanese rice against rice harvested in the United States, Australia, and many Asian countries based on δ^{13}C and δ^{18}O values.

2. Light element stable isotope ratios of rice harvested in Japan

δ^{18}O value of rice harvested in Okinawa Prefecture was higher, and δ^{13}C value of rice harvested in Hokkaido tended to be lower than those of rice harvested in other areas in Japan. δ^{13}C value of rice harvested in Kyushu tended to be higher whereas some of them fell in the range of the value of the rice harvested in Honshu and Shikoku. It is, therefore, considered to be difficult to discriminate geographic origin of rice within Japan by only light stable isotope analysis.

3. Analysis of change in stable isotope ratios of rice by cooking

No significant difference in δ^{18}O, δ^{13}C and δ^{15}N values was observed by the difference of washing water or cooking process in two-way analysis of variance (ANOVA) using all data of the raw rice, the washed rice and the cooked rice samples. δ^{15}N value of the rice cooked using tap water of Musashino City was, however, found to be significantly lower than those of rice cooked using other waters by one way AVOVA. Thus only δ^{18}O and δ^{13}C geographical database of raw rice is considered to be useful for geographic origin determination of boiled rice.