Epidemiological studies of food allergy and food-responsive enteropathy in dogs in Japan

（日本における犬の食物アレルギーと食事反応性腸症に関する疫学研究）

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GENERAL INTRODUCTION

Food allergy is one of the critical allergic diseases in dogs. Among food intolerance that is a general term for adverse reactions caused by ingesting food, food allergy is a reaction triggered by an immunological mechanism and is defined as all immune-mediated reactions that follow the ingestion of food. Food allergy is a non-seasonal skin disease and can be accompanied by chronic gastrointestinal symptoms regardless of the presence/absence of skin symptoms (Verlinden et al., 2006). Food allergy sometimes causes gastrointestinal symptoms, such as vomiting, loose stool and diarrhea (Ishida et al., 2004). It was reported that 60% of food allergy cases accompanied gastrointestinal symptoms (Loeffler et al., 2006a).

In contrast to food intolerance, which is caused by non immune-mediated mechanism, food allergy is categorized into the type I hypersensitivity related with histamine release from mast cells triggered by antigen-IgE antibody interaction, and the non IgE-mediated, type III and type IV, hypersensitivities (Verlinden et al., 2006). It was also reported that serum allergen-specific IgE levels did not always increase in dogs with food allergy, but dogs with atopic dermatitis tends to have allergen-specific IgE in their serum (Ishida et al., 2003). Furthermore, peripheral blood lymphocytes from dogs with food allergy showed proliferative response to food allergens *in vitro* (Ishida et al., 2003), suggesting the possibility of involvement of type IV hypersensitivity induced by T cells. Afterwards, this lymphocyte proliferative test became commercially available in Japan (Fujimura et al., 2011). This test is useful to help to identify causative food

allergens in dogs with food allergy, and furthermore, to monitor an extent of allergic reaction during the course of treatment (Fujimura et al., 2011). On the other hand, it was reported that incidence rate of food allergy was 25-27% of cases with allergic dermatitis (Picco et al., 2008), however, the immunological mechanisms, such as type I or type IV hypersensitivities, were not analyzed in their study. This means that there are still many unknowns regarding non IgE-mediated food allergy, and incidence rates for food allergy in dogs caused by non IgE-mediated type IV hypersensitivity have not reported yet. Therefore, in Chapter 1, we evaluated the prevalence of dogs that showed lymphocyte proliferative response among the dogs with allergic dermatitis.

Chronic Enteropathy (CE) is a general term for the disease showing chronic gastrointestinal symptoms including vomiting and diarrhea in dogs, and etiologically categorized into Antibiotic-responsive diarrhea (ARD) that reacts to antibiotic therapy, Food-responsive diarrhea (FRD) that reacts to dietary therapy, and Steroid-responsive diarrhea (SRD), sometimes called as idiopathic Inflammatory Bowel Disease (IBD) (Simpson & Jergens, 2011; Hall, 2011). In contrast to human medicine, in which IBD indicates two kinds of diseases, ulcerative colitis and Crohn's disease, the word ‘IBD’ in veterinary medicine is used synonymously with SRD and is defined as an idiopathic, chronic gastrointestinal syndrome characterized by mucosal inflammation (Jergens et al., 1992; Jergens, 1999). Therefore, IBD or SRD is diagnosed by the histopathological examination with a gastrointestinal endoscopy. Importantly, however, to diagnose IBD

or SRD, FRD is reliably ruled out by confirming no improvement of clinical symptoms by dietary therapy before performing the histopathological examination, because there was no difference in the histopathological results between FRD and SRD (Schreiner et al., 2008).

FRD includes food allergy triggered by an immunological mechanism and food intolerance triggered by a non- immunological mechanism (Gaschen & Merchant, 2011). Gastrointestinal symptoms of both diseases can be improved by a dietary change. Hence, FRD is considered as a chronic intestinal disease that can be improved by changing foods to a low-fat, high fiber (Leib, 2000) or hypoallergenic diet, e.g. hydrolysis, novel protein and amino acid diet (Marks et al., 2002; Nelson & Stookey, 1988). Because a great deal of efforts to select most appropriate foods needs to be required to diagnose FRD, there are few reports regarding the prevalence of AFR among chronic enteropathies in dogs. Swiss study showed that 55.7% (39/70) of cases with CE were food-responsive group and 30% (21/70) of them were not food-responsive and required steroid therapy (Allenspach et al., 2007) , but in Japan, the prevalence of AFR among the cases with CE has not been reported yet. For this reason, we investigated the percentage of AFR dogs to cases with CE in Japan in Chapter 2.

CHAPTER 1

The prevalence of dogs with lymphocyte proliferative responses to food allergens in canine allergic dermatitis

SUMMARY

The aim of the present study was to examine the correlation between the results of lymphocyte proliferative test (LPT) specific to food allergens and allergic skin diseases in dogs. Investigations were performed in 138 dogs with allergic skin diseases diagnosed in a private animal hospital. Of the 138 animals, 97 cases had positive reactions in LPT specific to food allergens. Of these 97 dogs, 67 animals were diagnosed with canine atopic dermatitis (CAD), but 30 dogs did not have IgE antibodies to environmental allergens. As 14 dogs out of 30 animals showed a positive result, 12 dogs underwent elimination diet trial based on the test results and all of them showed improvement in the pruritus score. Therefore, we conclude that LPT is effective diagnostic test for allergic skin disease. Results of the lymphocyte test are useful in the identification of food allergens for the elimination diet trial.

INTRODUCTION

Allergic skin diseases in dogs can be broadly classified into the following two groups: atopic dermatitis and food allergy. Canine atopic dermatitis (CAD) can be diagnosed either by intradermal skin test (IDST) or the presence of allergen-specific immunoglobulin E (IgE) to environmental allergens in the serum in combination with clinical symptoms described in Favrot's diagnostic criteria (Favrot et al., 2010). In cases where allergen-specific IgE is not detected in the serum, the disease is reclassified as atopic-like dermatitis (Halliwell, 2006). Over the years, the diagnosis of a food allergy is often challenging. Clinical symptoms of food allergy overlap with those of CAD (Chesney, 2002; Loeffler et al., 2006b). IDST or allergen-specific IgE test is neither sensitive nor specific enough to diagnose food allergy (Paterson, 1995; Jeffers et al., 1996a; Ishida et al., 2004; Olivry et al., 2007). One of the reasons for this is that food allergy occurs through the combined mechanisms of IgE-mediated (type I) and non-IgE-mediated allergic reactions. The lymphocyte proliferation test (LPT) is useful in the detection of non-IgE-mediated allergic reaction to food allergens in dogs (Ishida et al., 2004) . Lymphocytes of dogs that are sensitized to food allergens will proliferate in response to the specific allergens *in vitro* (Ishida et al., 2004) . Results of the LPT were consistent in dogs diagnosed with food allergy by food elimination and provocation tests where elimination of the food allergens improved the clinical symptoms of patients with reduced lymphocyte proliferative responses to the causative

allergens and vice versa (Fujimura et al., 2011). An allergen-specific LPT for food allergy (Ishida et al., 2004) has recently become commercially available in Japan (Fujimura et al., 2011). This study describes a retrospective analysis of the correlation between allergic skin diseases diagnosed in 138 dogs and the results of LPT specific to food allergen.

MATERIALS AND METHODS

We reviewed the medical records of dogs that visited the Primo Animal Hospital with the main complaint of pruritus between June 2008 and September 2010. Dogs diagnosed with ectoparasitic infestations, such as sarcoptic and demodectic mange, by general dermatological examinations were excluded from the study. In addition, dogs with pyoderma, fungal infections, Malassezia dermatitis, and flea allergy dermatitis were also excluded from the study as these skin conditions hinder the accurate assessment of pruritus. Cases with a differential diagnosis of atopic dermatitis that met the requirements of the Favrot diagnostic standards (Favrot et al., 2010) and have not had any prior treatment at the time of the examination were selected. Serum and whole blood samples were collected for the allergen-specific IgE test and LPT, respectively (Fig. 1).

Allergen-specific IgE was measured with a new commercially available quantitative fluorometric enzyme-linked immunosorbent assay (Animal Allergy Clinical Laboratories, Inc., Sagamihara, Kanagawa, Japan) (Okayama et al., 2011). The 40 allergens measured in this assay included 22 environmental allergens (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, flea, mosquito, cockroach, mugwort, ragweed, goldenrod, dandelion, daisy, orchardgrass, sweet vernal, timothy, rye, bermuda, Japanese cedar, birch, alder, *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, and *Penicillium notatum*). Each allergen was

prepared as described in previous report (Okayama et al., 2011). The allergen solutions were diluted at concentrations of 10-20 μg/ml of total protein and immobilized in 96-well plates. A combination of rat anti-mouse IgE antibody (R35-72, BD, Franklin Lakes, NJ, U.S.A) immobilized in the well plate and purified mouse IgE (C38-2, BD) with pre-determined concentration was used for the standard curve. To determine the background reaction, pooled serum of normal BALB/c mice and healthy beagles (Zenoaq Nihon Zenyaku Kogyo Co., Ltd.) were used after subtracting the background fluorometric titers. All the results were calculated according to the standard curve and expressed in a scale of ng/ml.

Using sera from 46 clinically healthy dogs, the cut-off value of serum IgE was determined to be 100ng/ml as reported (Okayama et al., 2011) and results above this value were considered as positive reactions.

The allergen-specific T lymphocyte reaction was measured with a commercially available system (Animal Allergy Clinical Laboratories, Inc.)(Fujimura et al., 2011). Briefly, peripheral blood mononuclear cells (PBMCs) were purified and cultured in RPMI-1640 medium (Sigma, St Louis, MO, U.S.A.) containing 10% fetal bovine serum (Equitech-Bio Inc., Kerrville, TX, U.S.A.) and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) (Sigma). Each of the 18 food antigens used in the allergen-specific IgE test were added to the PBMCs and incubated at 37ºC with 5% C02 in air for 4 days. After that, the cultures were continued for 3 days with the addition of human recombinant Interleukin-2 (PeproTech Inc., Rocky Hill, NJ, U.S.A.).

Concanavalin A (Sigma) was used as positive controls to examine the capability of cell proliferation in the culture. As described in the previous reports (Masuda & Yasuda, 2008; Fujimura et al., 2011) , cultured cells were finally stained with Alexa 647-labeled anti-canine CD4 antibody (Serotec Ltd, Oxford, UK) and PE-labeled anti-human CD25 antibody, ACT-1 (DAKO A/S, Denmark) for the detection of CD4+/CD25low cells, which indicates the proliferative fractions of lymphocytes in response to food allergens. The values of the cells cultured without the protein extract were subtracted as a background from each value of those cultured with different food proteins. The cut-off value of the percentage of CD4+/CD25low cells in CD4+ lymphocyte in the lymphocyte proliferation test was 1.2%, as determined by samples from clinical healthy dogs (Fujimura et al., 2011) and any value above this was considered as a positive result. All aspects of this study were approved by the Animal Care and Use Committee of Zenoaq Nippon Zenyaku Kogyo.

For elimination diet trials, based on the results of LPT, the optimal diet, either Hill's Prescription Diet z/d Canine ULTRA Allergen-Free (Hill's Colgate (Japan) Ltd., Tokyo, Japan), lams Veterinary Formulas Skin and Coat Response FP dry or KO dry (P&G Japan, Tokyo, Japan), was selected and was fed by owners at home. During the elimination diet trials, no diet was fed other than water and the selected optimal diet. Changes in clinical condition were carefully observed and noted by the owners. Pruritus was assessed by the owners using a vertical visual analog scale with grade descriptors

(Rybnicek et al., 2009) at the first visit and at least 2 weeks after elimination of the diet trial.

The chi-squared test was used to evaluate the difference in the number of positive cases of each allergen- specific serum in the LPT. P values of less than 0.05 were considered as significant. Statistical analyses were performed with Microsoft Excel.

RESULTS

A total of 138 dogs met the inclusion criteria. Of the 138 dogs, 83 (60.1%) were intact or neutered females, and 55 (39.9%) were intact or castrated males. In total, 36 breeds of dog were represented, and, of these, the most commonly represented breeds were Miniature Dachshund (n = 23), Toy Poodle (n = 16), French Bulldog (n = 11), Shih Tzu (n = 10), Shiba Inu (n = 8), Chihuahua (n = 7), Miniature Schnauzer (n = 7), Miniature Pinscher (n = 6), and crossbreed (n = 11). The age of the dogs at admission ranged from 6 months to 14 years.

Of the 138 cases of allergic skin diseases examined, 104 (75.4%) had positive reactions in the IgE test that were specific to environmental allergens and 34 (24.6%) did not (Fig. 2a). These positive cases were diagnosed with CAD, but more than half of these cases (67 cases; 64.4%) also had positive reactions to food allergens in the LPT. Moreover, 30 of the 34 cases that did not have positive reaction in the IgE test specific to environmental allergens had positive reactions to food allergens in the LPT. The chi-square test showed that soybean was the most significant cause of food allergy ($p < 0.01$, Fig. 2b) among the food allergens that induced an increase in LPT against the food allergen. This was followed by rice, potato, and wheat.

Among the 30 cases that had positive lymphocyte proliferative reaction to food allergens in non CAD group (Fig. 2a, bottom column), 16 dogs also had positive reaction to food allergens in the IgE test, but 14 dogs did not. Out of 14 dogs showing

only positive reaction to food in LPT, complete medical information was available in 12 cases for following-up investigations of the clinical symptoms after elimination diet trials to be carried out. All 12 dogs showed decrease in pruritus score after elimination diet trial without any other medication (Fig. 2c).

DISCUSSION

Both IgE and non-IgE-mediated hypersensitivity reactions are involved in food allergy in dogs (Ishida et al., 2004) . In order to diagnose food allergy and identify the causative food allergens, canine patients conventionally undergo elimination diet trials and provocation tests. However, these tests are not typically performed in veterinary clinics due to the cumbersome nature. Hence, it has been difficult to identify the causative food allergen in these cases. It is not until recently that a commercially available allergen-specific LPT in Japan has eased the process of diagnosis of allergic skin diseases (Fujimura et al., 2011) . However, there are no reports on the prevalence of cases with LPT-positive reaction to food allergens in dogs. In this retrospective study, we showed that an unexpectedly large proportion of dogs with allergic skin diseases (97/138; 70.3%) showed positive reactions in the LPT to food allergens. This finding may indicate that a high number of dogs with allergic skin diseases have unexpected allergic reactions to food allergens and that the existence of food allergen-sensitized lymphocytes in the peripheral blood may be the cause in cases of food allergy that were not diagnosed by the IgE-specific diagnostic technique. Furthermore, most of cases which were categorized to non CAD showed positivity to the food allergen in the LPT. Recently, dogs showing allergic skin diseases without elevated levels of IgE that are specific to environmental allergens have been reclassified as atopic-like dermatitis (Halliwell, 2006) . The cases (30 dogs) that were identified as LPT-positive without

positive reactions to environmental allergens in the IgE tests in this study might be those cases classified as atopic-like dermatitis and this needs to be elucidated in another study.

Elimination diet trial and provocation tests based on the results of the LPT were only performed in some cases as shown in Fig. 2c. Even though these clinical tests are known as the gold standard in the diagnosis of food allergy in dogs, these tests, especially the provocation test, are often not performed due to owners' reluctance. Previous report (Ishida et al., 2004) indicated that LPT is a good test to identify the causative food allergen. The high prevalence of LPT positive reactions in dogs with allergic skin diseases in this report prompted us to correlate the LPT results and the results of elimination diet trial. As expected, elimination diet trial performed based on the results of LPT alleviated the allergic skin disease.

We revealed that the high prevalence of LPT positive reactions in dogs with allergic skin diseases. This suggests that LPT can be used as an effective additional diagnostic tool for canine allergic skin disease.

FIGURES

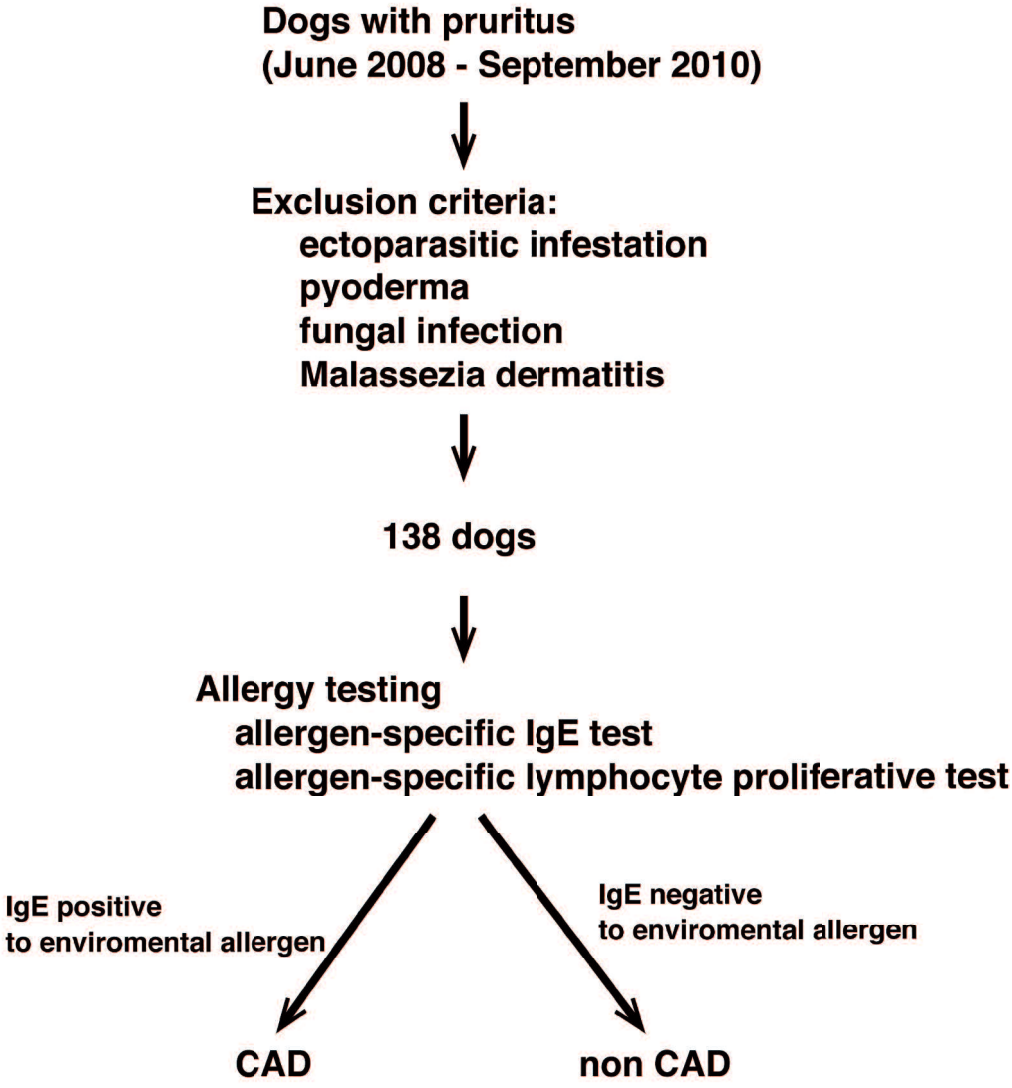

Fig. 1. Study design.

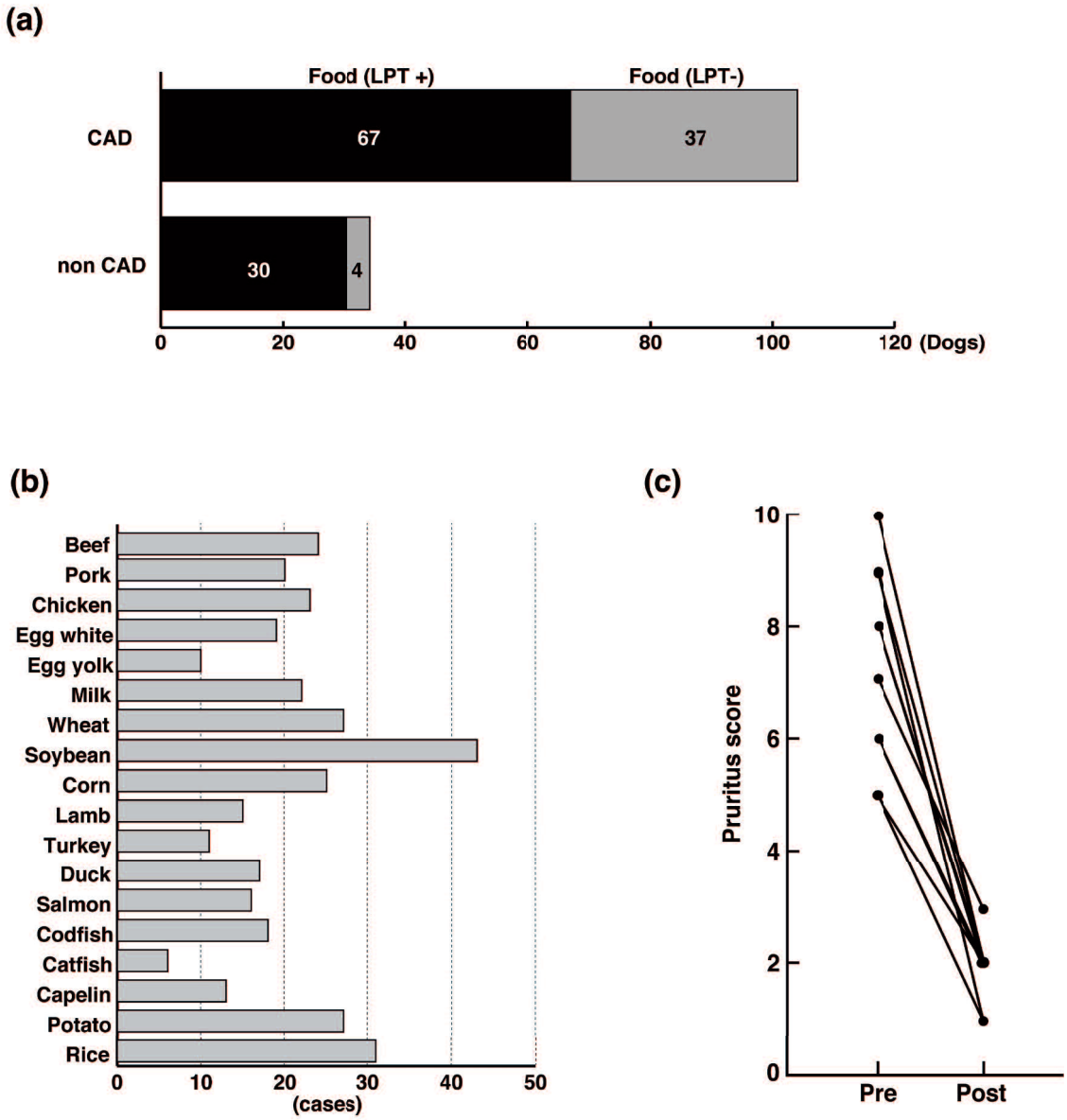

Fig. 2. The incidence of environmental allergen- and food allergen-positive cases: (a) The upper and lower bars indicate the numbers of cases with allergen-specific Immunoglobulin E (IgE)-positive canine atopic dermatitis (CAD) and those that were negative (non-CAD), respectively, to environmental allergens. The black bar indicates the number of cases that showed positive reaction to food allergens in the lymphocyte

proliferation test (LPT), (b) Food allergens identified by the LPT, (c) Changes in pruritus score in 12 dogs between before and after food elimination test.

CHAPTER 2

Prevalence of food-responsive enteropathy among dogs with chronic enteropathy in Japan.

SUMMARY

There have been limited reports on the prevalence of adverse food reactions among dogs suffering from chronic enteropathy (CE) in Japan. We examined the prevalence and histological features of food-responsive enteropathy (FRE) in a total of 32 dogs with history of CE. Fourteen of 18 cases (56.2%) diagnosed as FRE had lymphocytic-plasmacytic enteritis or eosinophilic enteritis by histopathological examination. Characteristic histopathological changes indicating FRE were not identified in 18 cases, though 4 cases did not show any abnormalities.

INTRODUCTION

Chronic enteropathy (CE) in dogs includes antibiotic-responsive diarrhea (ARD) responding to antimicrobial therapy, adverse food reactions (AFRs) responding to diet therapy and inflammatory bowel diseases (IBDs) (Hall, 2011; Simpson & Jergens, 2011) . Generally, IBD is diagnosed based on the detection of inflammation in the intestines identified by histopathological examination. However, in order to accurately diagnose IBD, it is necessary to completely rule out ARD and AFRs by performing multiple antimicrobial and diet therapies. A previous report indicated that 55.7% (39/70) of dogs with CE responded to dietary changes (food responsive (FR)), while 30% (21/70) was found to be responsive to steroid treatment (ST) where steroid is needed for the alleviation of clinical symptoms(Allenspach, Wieland, Gröne, et al., 2007) . Even though the author did not find any histopathological differences between dogs in the FR and ST groups, the results suggested that the canine chronic enteropathy activity index (CCECAI) is a good diagnostic indicator for CE, whereas negative prognostic factors for CE include high histopathological scores in the duodenum, and low cobalamin and albumin serum levels (Allenspach et al., 2007) . Up-to-date, there are no reports on the prevalence and clinical features of AFRs among dogs diagnosed with CE in Japan.

Specially formulated diets used to eliminate AFRs in dogs suffering from CE are often selected based on information from a list of previously exposed food.

Although Allenspach et al. used Purina L/A salmon and rice in all the cases to eliminate AFRs (Allenspach et al., 2007), it is unclear whether only one diet is adequate for this purpose. This suspicion was raised as it is widely known that selection of food allergens to be removed from diets in cases of food allergy is a particularly difficult task (Jeffers et al., 1996b) . Nevertheless, it has been recently reported that the Lymphocyte Proliferative Test (LPT) is helpful in selecting the most suitable food allergen that should be eliminated from the diet of dogs with food allergy (Fujimura et al., 2011; Kawano et al., 2013; Suto et al., 2015) . The objective of this study is to find out the prevalence of AFRs in dogs diagnosed with CE in Japan. Moreover, histopathological examinations were performed with samples obtained through endoscopy in order to find out if there is a connection between the AFRs and the histopathological diagnosis.

MATERIALS AND METHODS

Information of dogs that visited 10 animal hospitals (Primo Animal Hospital Nerima-Animal Allergy Medical Center, Yuki Animal Hospital, Urayasu Central Animal Hospital, Japan Animal Medical Center, Ogawa Canine & Feline Hospital, Kannai Animal Clinic, AEON Animal Medical Center, Hiratsuka Animal General Medical Center, Tokyo University of Agriculture and Technology, and Yamaguchi University) from August 2006 to February 2013 with the primary complaint of chronic digestive symptoms for over three weeks, including vomiting, diarrhea and weight loss, were compiled. Among all the cases, a total of 32 dogs meet the following requirements as criteria of study inclusion: 1) no immunosuppressive agents used for 2 weeks prior to enrollment into this study; 2) Endoparasitic infections were ruled out by fecal examinations performed by direct smear and zinc sulfate flotation; 3) dogs were treated with antibiotics (metronidazole 15–30 mg/kg BID, ampicillin 10–20 mg/kg BID or fluoroquinolone 5 mg/kg SID) for at least 2 weeks to rule out antibiotic-responsive enteropathy; 4) mucous membrane samples were collected from the stomach and duodenum through post-fast endoscopic examinations for histopathological examinations; and 5) elimination diet trials were performed for at least 10 days. The dogs that improved the gastrointestinal signs were includes in the food-responsive group. Elimination diet was selected individually based on the result of LPT (Animal Allergy Clinical Laboratories Inc., Sagamihara, Japan), which measured the *in vitro*

proliferative responsiveness of lymphocytes to the following 18 possible food allergens: beef, pork, chicken, egg white, egg yolk, milk, wheat, soybean, corn, mutton, turkey, duck, salmon, codfish, catfish, capelin, potato and rice. In cases where the lymphocyte proliferative index exceeded 1.2%, the food was considered as a causative allergen and excluded from the diet trial (Fujimura et al., 2011) . Glucocorticoid (prednisolone 0.5–2 mg/kg/day) was administered to dogs that showed no clinical improvement by food elimination tests. If clinical improvement was observed after glucocorticoid therapy, the dogs were included in the steroid-treatment group. Furthermore, serum albumin concentration was measured in all cases, and level of 20 g/L or lower was defined as hypoalbuminemia. The severity of clinical presentations was determined with CCECAI (Allenspach et al., 2007). CCECAI is a clinical scoring index, which is the sum of score assessing nine aspects of dogs suffering from CE, such as activity, appetite, vomiting, stool consistency, frequency of bowel movement, weight loss, albumin concentration, ascites fluid, subcutaneous edema and itching.

The correlation between hypoalbuminemia and the food responsive/steroid-treatment groups was analyzed statistically with the Fisher's exact test. Statistical processing was performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). Data were considered as significant different when $P<0.05$.

RESULTS

The clinical characteristics of all 32 dogs are described in Table 1. The average age of the dogs enrolled in the present study was 5.33 years (range: 4 months −13 years), and the breed of dogs in this study included Toy Poodle (n=6), Shiba (n=3), Papillon (n=3), Pomeranian (n=3), Shih Tzu (n=2), Miniature dachshund (n=2), Chihuahua (n=2) and Mongrels (n=2), and one Jack Russell terrier, Yorkshire terrier, Boston terrier, French bulldog, Labrador retriever, Shetland sheepdog, Welsh corgi, English Cocker Spaniel and American Cocker Spaniel. From the LPT results, 31 of 32 cases had positive lymphocyte proliferative response to more than 2 food allergens (Table 2), and one dog (case no.1) did not show reactivity to any food allergens.

After the dogs were fed with elimination diets based on the LPT results, 18 of 32 dogs (56.2%) were assigned as food-responsive, while the remaining 14 dogs (43.8%) were only responsive to steroid treatment (Table 1). Elimination diets that improved the clinical signs of dogs in the food-responsive group were: ANALLERGENIC (n=8), Select Protein (D&T) (n=1) (Royal Canin Japon Inc., Tokyo, Japan), z/d ULTRA Allergen-free (n=2), w/d (n=1) (Hill's-Colgate (Japan) Ltd., Tokyo, Japan), Amino Protect Care (n=2) (Nosan Corporation, Yokohama, Japan), D Assist KO Select Protein (n=1), D Assist FP Select Protein (n=1), (Eukanuba, Cincinnati, OH, U.S.A.), and home-made diets (n=2) (Table 2). The results in the present study showed

that the percentage of dogs diagnosed with CE in Japan that clinically improved after elimination diets is 56.2% (18/32).

The results of the histopathological examinations from all the cases were lymphocytic-plasmacytic enteritis (LPE; n=25), eosinophilic enteritis (n=3) and minimal change (n=4) (Table 1). In those cases diagnosed as LPE, 13 (52%) were in the steroid-treatment group, and 12 (48%) were in the food-responsive group. From this result, there are no specific histopathological changes consistent with a diagnosis of food-responsive enteropathy.

Ten out of 32 cases showed hypoalbuminemia. Out of the 10 cases, 8 (80.0%) were in the steroid-treatment group, and 2 (20.0%) were in the food-responsive group. Although the percentage of steroid-treatment group among all cases was 43.8%, the percentage of steroid-treatment group among all cases with hypoalbuminemia increased to 80.0%. Sensitivity and specificity of hypoalbuminemia in the steroid-treatment group were 57.1% (95% confidence interval 0.29–0.82) and 88.9% (95% confidence interval 0.65 to 0.99), with likelihood ratios of 5.14. Statistical analysis with Fisher's exact test showed significant correlation between hypoalbuminemia and each of the groups (P=0.0084) and a high odds ratio of 10.67 in cases with hypoalbuminemia compared with those at normal albumin level.

The CCECAI value is a scoring index for CE in dogs. The average CCECAI in all the cases was 7.4 (range: 1–13) (Table 1). The CCECAI value in the steroid-treatment group (8.6 ± 3.3 (mean ± SD)) was higher than that in the food

responsive group (6.4 ± 2.8 (mean ± SD)), but there were no statistically significant differences (P=0.096).

DISCUSSION

The results in the present study showed that the percentage of dogs diagnosed with CE in Japan that clinically improved after elimination diets is 56.2% (18/32). A previous study conducted in Switzerland(Allenspach et al., 2007) suggested that the percentage of dogs suffering from CE that clinically improved after elimination diets stood at 55.7% (39/70).

As shown in this study, a previous study on histological evaluation in dogs diagnosed with CE also showed that there was no difference between steroid-responsive and food-responsive diarrhea (Allenspach et al., 2007; Schreiner et al., 2008). Moreover, another study also showed that most of dogs with diet-responsive chronic enteropathy had been diagnosed as a LPE by endoscopic examination (Walker & Takano, 2013) . Thus, as supported from our result in this study, food-responsive enteropathy (or AFRs) and steroid-treatment enteropathy (IBD) cannot be discriminated based on histopathological results. Cases of ARD and AFRs that would respond to antibiotics and dietary change might be mistakenly treated with steroid if they are not properly ruled out first. On the other hand, all four cases showing no abnormality in the histopathological examinations were in the food-responsive group. Even though four cases were not enough to make a solid conclusion, it suggests that recommending dietary management first is appropriate if no abnormality was observed in the histopathological examinations. Among the three cases of eosinophilic enteritis, one

was in the steroid-treatment group, and two were in the food-responsive group. Clinical importance of eosinophilic infiltration in intestinal mucosa was not clarified well. However, Walker et al. showed a tendency for eosinophilic infiltration in food-responsive enteropathy, and the mean density of eosinophils seemed to decrease after treatment (Walker et al., 2013) . From previous and our studies, the relationship of eosinophilic infiltration and pathogenesis of food-responsive enteropathy was not yet clarified.

In the present study, the diets of the dogs were changed based on the LPT results. LPT may be helpful to find the suitable elimination diet for the dogs with food-responsive enteropathy. However, the positivity in LPT did not necessarily indicate that the dog had the food-responsive enteropathy, because 31 of 32 cases had at least one positivity to food allergen by LPT and about half of the dogs did not improve the symptoms after diet changes.

A weak point in this study was that the reproducibility of the clinical results was not confirmed by performing food provocation tests after the improvement of clinical symptoms. Therefore, it remains unclear whether these dietary changes can suppress the inflammatory responses through immunological mechanisms. Furthermore, the role of food allergens as one of the etiologies of CE has not yet been established. It is well known that food allergy in dogs occurs in the presence of two types of pathological conditions, the IgE-mediated and the non IgE-mediated hypersensitivity (Ishida et al., 2004; Kawano et al., 2013; Suto et al., 2015) . In cases of the non

IgE-mediated hypersensitivity, it has been indicated that LPT can be useful in identifying the possible causative antigens. Instead of focusing on the pathological mechanism of AFRs or IBD, demonstrating the role of LPT in AFRs seems far more important as LPT can also be used to identify food allergens in treating cases of food-responsive enteropathy.

Ten out of 32 cases showed hypoalbuminemia. Out of the 10 cases, 8 (80.0%) were in the steroid-treatment group. These results suggested that immunosuppressive agents, such as steroid, will be required, in addition to dietary management, in cases of CE with hypoalbuminemia.

The CCECAI value in the steroid-treatment group (8.6 ± 3.3 (mean ± SD)) was higher than that in the food responsive group (6.4 ± 2.8 (mean ± SD)). A previous study (Schreiner et al., 2008) reported that the steroid-responsive group showed a significantly higher CCECAI value (P=0.05) as compared to the food-responsive group. Mean CCECAI values of food-responsive group in our study and their study were quite similar (6.4 versus 6.0, respectively), but the values in the steroid-treatment group were different (8.6 versus 10.0, respectively). Mean CCECAI value in the steroid-treatment group in our study was lower than the other study, and this possibly contributed to no significance between the two groups in our study. Unfortunately, we were unable to identify the reason behind the difference between the CCECAI values of the studies. Larger studies with more complex analyses might be required in the future.

In conclusion, the present study examined dogs from multiple animal hospitals in Japan that were diagnosed with CE, and the results revealed that 56.2% (18/32) of them had food-responsive enteropathy. In particular, cases where histopathological examinations appeared to be normal and hypoalbuminemia was not observed had a higher probability of being diagnosed with food-responsive enteropathy. Therefore, it is recommended that food allergens to be avoided in the elimination diet should be selected based on the results of LPT and subsequent elimination diet therapy be carried out to prevent any unnecessary use of immunosuppressants.

Table 1. Summary of information and results

Case No.	Age (Y)	Sex	Breed	Hypoalbuminemia (g/L)	CCECAI value	Histopathological diagnosis	Elimination diet	Group
1	6	Spayed female	Pomeranian	No	7	Nil	Select Protein (D&T)	Food-responsive
2	5	Castrated male	Mongrel	No	11	Nil	D Assist KO Select Protein	Food-responsive
3	5	Castrated male	Chihuahua	No	6	Nil	ANALLERGENIC	Food-responsive
4	7	Castrated male	Toy poodle	No	11	Nil	ANALLERGENIC	Food-responsive
5	5	Castrated male	English Cocker Spaniel	No	7	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Food-responsive
6	13	Intact male	Mongrel	No	8	Lymphocytic-plasmacytic enteritis	Home-made food (ostrich meat, pumpkin)	Food-responsive
7	0.3	Intact female	Shih Tzu	No	4	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Food-responsive
8	6	Spayed female	Labrador retriever	No	6	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Food-responsive
9	0.7	Castrated male	Papillon	No	5	Lymphocytic-plasmacytic enteritis	Amino Protect Care	Food-responsive
10	8	Intact male	Shih Tzu	No	6	Lymphocytic-plasmacytic enteritis	Home-made foods (chicken, Somen)	Food-responsive
11	5	Spayed female	Toy poodle	No	1	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Food-responsive
12	11	Spayed female	Toy poodle	Yes (15)	5	Lymphocytic-plasmacytic enteritis	z/d ULTRA Allergen-free	Food-responsive
13	2	Spayed female	Welsh corgi	No	10	Lymphocytic-plasmacytic enteritis	w/d	Food-responsive
14	5	Spayed female	Jack Russell terrier	No	4	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Food-responsive
15	3	Spayed female	Shiba	No	5	Lymphocytic-plasmacytic enteritis	Amino Protect Care	Food-responsive
16	8	Spayed female	Papillon	No	3	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Food-responsive
17	12	Castrated male	Papillon	Yes (17)	10	Eosinophilic enteritis	z/d ULTRA Allergen-free	Food-responsive
18	4	Castrated male	Shiba	No	7	Eosinophilic enteritis	D Assist FP Select Protein	Food-responsive
19	1	Spayed female	Toy poodle	No	4	Eosinophilic enteritis	ANALLERGENIC	Steroid-treatment
20	6	Spayed female	Pomeranian	No	13	Lymphocytic-plasmacytic enteritis	d/d salmon & potato	Steroid-treatment
21	1	Intact female	Shiba	Yes (15)	8	Lymphocytic-plasmacytic enteritis	Amino Protect Care	Steroid-treatment
22	2	Intact female	Toy poodle	Yes (13)	9	Lymphocytic-plasmacytic enteritis	Amino Protect Care	Steroid-treatment
23	7	Spayed female	Shetland sheepdog	Yes (17)	3	Lymphocytic-plasmacytic enteritis	D Assist KO Select Protein	Steroid-treatment
24	1	Intact male	Yorkshire terrier	No	12	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Steroid-treatment
25	9	Intact male	Pomeranian	Yes (15)	13	Lymphocytic-plasmacytic enteritis	z/d ULTRA Allergen-free	Steroid-treatment
26	2	Spayed female	Chihuahua	No	7	Lymphocytic-plasmacytic enteritis	z/d ULTRA Allergen-free	Steroid-treatment
27	9	Intact male	Miniature dachshund	Yes (13)	11	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Steroid-treatment
28	7	Intact female	Boston terrier	Yes (11)	10	Lymphocytic-plasmacytic enteritis	Amino Protect Care	Steroid-treatment
29	5	Intact male	French bulldog	Yes (10)	8	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Steroid-treatment
30	6	Intact female	Miniature dachshund	No	11	Lymphocytic-plasmacytic enteritis	D Assist KO Select Protein	Steroid-treatment
31	2	Castrated male	Toy poodle	No	4	Lymphocytic-plasmacytic enteritis	z/d ULTRA Allergen-free	Steroid-treatment
32	8	Spayed female	American Cocker Spaniel	Yes (12)	8	Lymphocytic-plasmacytic enteritis	ANALLERGENIC	Steroid-treatment

Lymphocyte Proliferative Index (%)																Group
hicken	Egg White	Egg Yolk	Milk	Wheat	Soybean	Corn	Mutton	Turkey	Duck	Salmon	Codfish	Catfish	Capelin	Potato	Rice	
0	0	0	0	0.4	0	0.3	0.1	0	0	0	0	0	0.2	0.5	0.3	Food-responsive
1.9	2.6	1.3	3	0.5	1.6	1.2	4.2	1.2	0	0	0	0.7	2.2	0.2	1.7	Food-responsive
5.2	3.6	2.3	2.7	0.8	3.6	4.1	4.6	2.9	3.1	2.7	2.1	1.2	2.8	2.6	0.3	Food-responsive
1.8	0	0	0	0	2.7	0	0	0	0	4.7	0	0	0	0	0	Food-responsive
1.6	1.3	1	0.3	1.9	3.6	1.3	1.2	0.9	0.7	1.4	3.1	0.7	1.1	5.5	5.7	Food-responsive
0.4	0	0.4	0	1.8	2.3	0	0	0	0	0	0.3	0.4	0	1.6	0.1	Food-responsive
0.6	0.3	0.4	2.8	1.6	1.2	0.1	1.4	0.4	0.4	0.3	1.2	0.2	0.4	5.5	2.5	Food-responsive
2.2	0	0	1.4	2.3	3.7	1.4	4.6	2.5	5.8	2.2	4.7	2.6	2.5	3.4	3	Food-responsive
3.1	1.2	4.7	4.1	2.4	1.2	0	1.4	1.7	1.7	3.8	1.5	2.7	1.9	2.9	0	Food-responsive
2	3.6	0.4	3	5.2	3.8	3.2	2.6	2.2	0	0.8	0	1.4	0	0	0.4	Food-responsive
0.3	1.6	0.5	0.8	0.6	1.6	0.6	1	0	0	0	0.6	0	0.6	0.7	0.7	Food-responsive
0.2	0.2	0.2	0.1	1	2.1	1.1	0	0.1	0	0	1.2	0.3	2.1	4.9	1.7	Food-responsive
0	0	0.2	0.4	4.6	1.5	0.5	0	0	0	0.2	1.6	1.2	1.3	4.2	3	Food-responsive
0	2.8	0.6	0.8	0.1	3.6	4.3	0	0	0.4	2.3	0	0	1.5	5	1.5	Food-responsive
2.4	0	3.6	0	0	0	0	0	0	0	1.3	2.4	0	0	0	2	Food-responsive
0.2	0.8	0.5	0.8	3.1	0.3	0.3	0.5	0.3	0.8	0.6	2.9	1.4	1.1	6	2.9	Food-responsive
0.7	0.3	0.8	0	1.5	1.5	1.2	0	0.3	0.5	0.5	0.4	0.7	0.1	3.7	3.1	Food-responsive
2	0.7	1.9	2.5	3.2	1.7	0	1.6	1.2	4.8	1.1	2	1.7	1	4.9	3.9	Food-responsive
1.1	1.1	0.8	0.8	0.9	1.4	0	1	1.4	2.2	0.6	0.6	1.2	0	0	0.8	Steroid-treatment
0	0.3	0.3	1	0.9	1	1.5	0.3	0.4	0.2	0	0.8	3	1.6	4.8	1.9	Steroid-treatment
0	0.6	0	0	0.4	2	2	0	0	0	0	0.2	0	0	1.4	1.1	Steroid-treatment
0	0	0	0	1.3	5.7	1.2	0	0	0.6	0	0.9	0	0.1	1.8	1.3	Steroid-treatment
0	1.2	0.2	0.7	0	1.2	0.8	0	0.2	1.2	0.1	1	1.5	1	0.9	1.5	Steroid-treatment
0.2	0.2	0	0.8	0.8	2.2	1.2	0	0	0.5	0	0	0	0	1	0.5	Steroid-treatment
0	0	0.9	0	0.8	0.4	0	1.2	1.8	0	0	0.2	0	0	0.3	1.3	Steroid-treatment
0.3	0	0	0	1.3	0.5	0	1.6	0.6	0	0.7	1.2	0.3	1.4	2.6	1.5	Steroid-treatment
1.4	0.7	0.5	0.3	4	3.3	0	0.4	0.9	1.2	0.9	1.2	0.9	0.2	3.5	4.7	Steroid-treatment
1.9	1.2	1.2	0.3	2.7	4.4	4	0	3.4	0.1	0	2.4	0	0.4	4.8	3	Steroid-treatment
0.6	0.2	0.4	0.3	2.7	1.4	1	1.1	0.2	1	1.4	2.9	1	1.4	2.5	3.8	Steroid-treatment
0.4	0.3	1.3	0	5.7	4.8	0.8	1.5	1.8	1.5	1.5	3	1.2	3	5	4.2	Steroid-treatment
0	0.1	0	0	1.7	0.6	0.8	0	0.1	0	0	1.2	0	0.4	1.6	3.2	Steroid-treatment
1.2	1.5	0.8	0.3	0.2	3.5	2.2	0.2	0.7	0	0	0	0	0.3	0	0.7	Steroid-treatment

ılt of LPT (%). Unshaded and shaded columns indicate "negative" and "positive", respectively.

CONCLUSION

In the medical field, food-related allergic diseases are categorized into IgE-mediated, non-IgE-mediated and mixed types, and gastrointestinal allergy is a general term for diseases caused by food allergens and accompanied with gastrointestinal symptoms (Järvinen & Nowak-Węgrzyn, 2013; Mehr et al., 2014). In canine food allergy, two types of hypersensitivities, IgE-mediated type I hypersensitivity and non IgE-mediated type IV, were reported(Ishida et al., 2004; Kawano et al., 2013), and the lymphocyte reaction test is reported to be useful to identify the causative food allergen for non IgE-mediated hypersensitivity. In Chapter 1, it was proved that 70.3% (97/138) of dogs with allergic skin diseases showed lymphocyte proliferative response against food allergens. This result suggested that many cases with canine food allergy is not caused by type I hypersensitivity but by non IgE-mediated type IV hypersensitivity.

As shown in Chapter 1, food allergy may be much more prevalent than previously considered. This means that some of the dogs diagnosed as an atopic dermatitis might have simultaneously food allergy in clinical setting. For the treatment of canine atopic dermatitis, new therapies have become available in recent years, e.g. immunomodulator therapy using IFN-gamma, molecularly targeted therapy using oclacitinib and allergen-specific immunotherapy, in addition to conventional treatments using glucocorticoid and cyclosporine. Most principal factor in the success of those new treatments is to diagnose accurately canine atopic dermatitis by ruling out food allergy,

as well as ectoparasites (e.g. flea, scabie and demodex folliculorum), pyoderma and fungal infections. Especially, diagnosis of food allergy by exclusion is most difficult part. If diagnosis of food allergy is overlooked in dogs with atopic dermatitis, clinical symptoms may not be controlled well, and sometimes, administration of unnecessary drug may be required, hence food allergy should be accurately diagnosed by appropriate selection of hypoallergenic diet.

In Chapter 2, I proved that, among 32 dogs diagnosed with chronic enteropathy in multiple animal hospitals, 56.2% (18/32) of them had Food responsive enteropathy and 43.8% (14/32) of them had steroid responsive enteropathy. This result was quite similar to Swiss' study. However, in my study, in order to diagnose Food responsive enteropathy, the optimal elimination diet was selected based on the results of allergen-specific IgE quantitative test and lymphocyte reaction test, and it makes much easier to select elimination diet and then improve dogs' clinical symptoms in relatively short term period. No one reported that food responsive enteropathy was caused by type IV hypersensitivity, and was diagnosed by lymphocyte proliferative test, however results in Chapter 2 support this hypothesis. I would like to expect this hypothesis may be proven in near future.

According to Chapter 1 and Chapter 2, it was proved that food-responsive disease (food allergy or food responsive enteropathy) were quite popular in canine disease, and ruling out or diagnosing food-responsive disease (food allergy or food responsive enteropathy) are extremely important in the canine cases showing dermatitis

or gastroenteric symptoms). Hopefully, this thesis would help to improve the diagnostic rate of canine allergic diseases in the future.

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