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Prevalance of presence of immunoglobulin E (IgE) against cross-reactive carbohydrate determinants (CCD) and impact of CCD inhibitor in multi-positive *in vitro* seasonal allergy tests in dogs and cats from July 2017 to June 2018 - a retrospective study

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1. Introduction

Atopic dermatitis (AD) is a common chronic, pruritic, inflammatory and genetically predisposed skin disease often seen in veterinary clinical practice which is mostly associated with generating of immunoglobulin E (IgE) against environmental allergens (Halliwell 2006, Marsella and Girolomoni 2009). Canine AD has been recommended as an animal model for human AD due to high clinical, immunological, pathological and histological similarities (Marsella and Girolomoni 2009, Mineshige et al. 2018). The clinical and historical features in cats are strikingly different to human and canine AD. Much less is known about the pathogenesis of feline AD. Head/neck pruritus accompanied with excoriations, self-induced symmetrical non-inflammatory alopecia, miliary dermatitis and eosinophilic granuloma complex are the non-specific cutaneous reaction patterns, which can be presented alone, or in different combinations. According to Hobi et al. 2011, non-flea, non-food induced hypersensitivity dermatitis (NFNFIHD) is preferred to Feline AD (FeAD), since importance and function of IgE in pathogenesis of disease are unclear yet. The diagnosis of AD is based on the history and clinical examination with exclusion of other pruritic skin diseases in both, dogs and cats (Hobi et al. 2011, Hensel et al. 2015). Allergen immunotherapy (AIT) is the only causal treatment option for canine and feline atopic patients (Saridomichelakis and Olivry 2016). Both intradermal skin test (IDST) and in vitro serum test are reliable diagnostic methods to identify offending allergens in patients with environmental allergies in order to formulate AIT (Mueller et al. 1999, Foster et al. 2003, Tarpataki et al. 2008, Hensel et al. 2015). The advantages of the serum test over the IDST are: detection of IgE antibodies without the necessity of sedation, less- painful/-time consuming procedure and minimal involvement of practitioner. It can be performed in patients with skin alterations due to inflammatory disorders like pyoderma or malassezia dermatitis; even with chronic cutaneous efflorescences like lichenification (Bevier et al. 1997).

Low specificity in pollen *in vitro* test was reported in dogs with a negative IDST and positive ELISA test (Codner and Lessard 1993). Therefore, false positive results were reported as a disadvantage of *in vitro* tests (Griffin et al. 1990, Miller et al. 1993). In order to avoid false positive results of *in vitro* allergy tests in human medicine, identification of allergy mimickers is crucial (Aalberse et al. 1981, Ebo et al. 2004). These positive results may be due to a low- molecular-weight carbohydrate with a glycoprotein structure, called N-glycan. This was identified in a wide variety of plant extracts (Aalberse et al. 2001, Altmann 2007). This N- glycan carrying α1,3-fucose and β1,2-xylose epitopes of plants are known as Cross-reactive carbohydrate determinants (CCD) (Faye et al. 1993, Altmann 2007, Altmann 2016). They are absent in mammalian tissue and can generate potent specific IgE antibodies against CCD (anti- CCD IgE) in more than 20% of human AD (Aberer et al. 2017). It needs to be clarified, why some patients generate anti-CCD IgE, while others do not (Wagner 2017). In most cases, binding of anti-glycan IgE with CCD epitopes did not elicit clinical symptoms (Altmann 2007). Though, one possible explanation is the need for IgE cross-linking with at least 2 epitopes on allergens. The monovalent structure of CCDs cannot supply the cross-linking and consequent release of the pro-inflammatory mediators (e.g., histamine, heparin) from mast cells (Van Ree and Aalberse 1999). This will result in the asymptomatic polysensitization causing false positive results of the *in vitro* pollen allergy tests. It can be also explained as an image of the anti-CCD IgE branch on a tree that shields traffic light (Altmann 2007).

Detection and blocking of anti-CCD IgE are important for the quality improvement of *in vitro* tests. This interference can be inhibited/suppressed by adding artificial glycoprotein extracts (Altmann 2007). The CCD inhibitor (CHO-blocker) is a highly purified synthetic glycoprotein, which does not contain any cross-reactive protein epitopes (Altmann 2016).

To the best of the authors' knowledge, until today, only two reports with rather low numbers of dogs were published on this topic in veterinary medicine and no study reported this item in cats. According to Levy and DeBoer, 24% of 38 canine sera contained anti-CCD IgE, while in the study from Gedon et al. anti-CCD IgE were identified in 38% from 31 canine sera from AD patients (Levy and DeBoer 2018, Gedon et al. 2019). Like in the human

studies, positive anti-CCD IgE sera have shown polysensitization in a vast majority of cases (Gedon et al. 2019). The lack of information about this subject in veterinary medicine is predominant.

Hypotheses:

1. Polysenzitisation in canine and feline seasonal allergy tests is highly prevalent and this should be shown on a large number of canine and feline serum samples.

2. Like in humans, CHO-blocker in seasonal allergy *in vitro* tests will block the false positive reactions in both species (dogs and cats)

The primary aim of this retrospective study was to (i) determine the prevalence of polysensitization in seasonal allergy *in vitro* tests in a large number of sera from dogs and cats and (ii) the evaluation of the impact of adding CHO-blocker in multi-positive test results in both species.

1. Methods and materials

2.1 Study Protocol

Retrospectively, data of 4614 dogs and 472 cats from seasonal *in vitro* allergy tests, received from July 2017 to June 2018 were analyzed. Analyses were performed separately for each month. Breeds, ages and genders for the individual tests in dogs and cats were documented.

2.2 Serum allergy tests

Canine and feline samples from different veterinary small animal practices and clinics across the EU countries, Switzerland, China and UK, with majority from Germany were

received. Seasonal allergy tests were performed by Laboklin veterinary diagnostic laboratory (Laboklin Laboratory for Clinical Diagnostics GmbH & Co. KG, Bad Kissingen, Germany). 16 common regional European allergens, such as 6-grass mix [orchard grass (*Dactylis glomerata*), timothy grass (*Phleum pratense*), meadow fescue (*Festuca pratensis*), perennial ryegrass (*Lolium perenne*), kentucky blue grass (*Poa pratensis*) and velvet grass (*Holcus lanatus*)], rye (*Secale cereale*), mugwort (*Artemisia vulgaris*), ragweed (*Ambrosia artemisiifolia*), English plantain (*Plantago lanceolata*), nettle (*Urtica dioica*) and sheep sorrel (*Rumex acetosella*), trees such as birch (*Betula populifolia*), hazel (*Corylus avellana*) and willow (*Salix caprea*) were included in the seasonal allergy panel. The result of immunologic responses for mugwort and ragweed (mugwort-ragweed), as well as for birch and hazel (birch-hazel) were determined together. The immunologic response to 6-grass mix extracts was shown as one allergen group. Serological testing was performed with the use of a commercially available allergen-specific IgE Fc-Ɛ receptor ELISA panel (Heska Allercept panel, Heska AG; Fribourg, Switzerland). Both, canine and feline sera were diluted 1:6 in the sample Tris-HCL-Diluent buffer (TRIS-saline 0.05 M, pH 7.5 containing 1% bovine serum albumin) and 100 µL incubated for 16-18 h at 4-8°C in allergen-coated ELISA wells. The plates were washed four times with Tris-HCL-Wash buffer, subsequently and then 100 µlof a 1:1000 dilution of Biotin- Fcε-R1-alpha reagent (10 ng/mL in Tris-HCL-Diluent Buffer) were incubated for 2 h (± 15 min) at room temperature (RT~22°C) and the plates were washed again. Afterwards, 100 µL of 1:1000 dilution of streptavidin-alkaline phosphatase-conjugate (125ng/mL in Tris-HCL-Diluent buffer) (Moss Inc.; Passadena, MD, USA) was added and incubated for 1h at RT. The plates were washed again, the reaction was revealed with 100 µL of Para-nitrophenyl phosphate (pNPP) (Moss Inc.) for 1h. The enzymatic reaction resulted in a colored product and was stopped with 50 µL of 50 mM L-cysteine. This color was stable for 24 h after adding the L-cystein solution. Subsequently, the reaction was read at 405 nm and the optical densities (OD) for each allergen were converted to HERBU (Heska Epsilon Receptor Binding Unit). The HERBU results were converted to 6 classes from 0 to five reaction classes (RC = 0 – 5). RC = 0 was considered negative and RCs ≥ 1 were considered positive. The tests with negative results (RC = 0) to all allergens were excluded from further analysis. Samples with at least one positive immunological response (RCs ≥ 1), to a minimum of one allergen, were included.

Both, canine and feline tests with positive reactions to the majority of allergens (RC ≥ 1) were considered polysensitized (Group A). All polysensitized samples were retested with a

modified blocking procedure and documented separately. These sera were incubated with Heska proprietary blocking solution (CHO-blocker) prior to the test (Heska AG; Fribourg, Switzerland). CHO-blocker inhibits binding of anti-CCD IgE with N-glycan structure of extracts (CCD) coated in ELISA wells. The blocker reagent consists of a combination of different plant glycoproteins containing a CCD composition specifically designed for the use in veterinary samples (Data not submitted).

In the end, group Ad (polysensitized), Bd (non-polysensitized) for dogs and Ac (polysensitized), Bc (non-polysensitized) for cats were created and used for further analyses. In all samples from group A (polysensitized) CHO blocking was performed and the ELISA testing was repeated afterwards. Results of allergy testing before- and after-incubation with CCD inhibitor were documented. To determine the impact of CHO-blocker, results prior- and post- blocking were evaluated for each tested allergen in 96 canine and 48 feline randomly chosen from the polysensitized sera (eight canine and four feline samples were randomly selected, analyzed and documented in each month). Discrepancies in immunologic reactions to each allergen were compared in these samples before- and after- blocking.

2.3 Statistical analysis

Statistical data analysis was performed using statistical software IBM SPSS statistics version. The samples were grouped into group A (Ad, Ac) and group B (Bd, Bc) based on the results (figure 1. and figure 2.). The positive and negative predictive value was calculated in 96 and 48 randomly selected samples from group A in dogs (Ad) and cats (Ac), respectively. The positive reactions which remained positive after blocking were considered true positives. The positive reactions which turned altered after blocking to negative reactions, were considered false positive. The negative reactions before blocking, that remained negative, were considered true negatives, and in case of changing to positive were considered false negative. Agreement prior- and post-blocking in this randomly selected group was measured with Cohen ‘s kappa. Values close to 0 were interpreted as poor agreement and values close to 1 indicated perfect agreement. The specificity and sensitivity of the test without blocking was calculated for cats and dogs separately.

3. Results

3.1 Results of seasonal allergy tests in dogs

A total of 4614 canine sera, 2098 female (1332 intact, 766 spayed), 2131 male (1453 intact, 678 neutered) and 385 unknowns were investigated. Due to negative results of the allergy test 1215 serum samples were excluded prior to analyses while 3399 samples were included.

The prevalences of groups Ad and Bd are shown for each month (Fig.1) from July 2017 to June 2018. Eight hundred eighteen (818, 24.07%) were evaluated as polysensitized (Ad) and 2581 (75.93%) samples were not (Bd).

The prevalence of polysensitization was lowest in February (n = 38; 17%) and Mai (n = 54; 18%), while the highest was detected in September (n = 101; 30%) and December (n = 56; 32%). From December to April, the numbers of submitted samples were lower in comparison to the rest of the year, but there was no difference in the presence of polysensitization in a seasonal dependent manner.

Ninty-six samples were randomly selected from the group Ad. The reactions for each allergen extract were converted to HERBU (Heska Epsilon Receptor Binding Unit). The HERBU results were converted to 6 classes from 0 to five reaction classes (RC = 0 – 5). In each sample, 8 RCs were shown. From 96 randomly selected serum samples, 768 reaction classes (immunologic reactions) prior- and post-blocking to each 8 allergens were evaluated, and the impact of CHO reagent is shown in Table 1, 2 and Table 3.

The total number of negative reactions (RCs = 0) is markedly increased post blocking from 38 (5%) to 391 (51%). Thus, from 730 (95%) positive reactions (RC $\geq$ 1), after adding CHO reagent solution, 356 (46%) RCs were completely inhibited (RCs = 0). Three hundred seventy-seven (377, 49%) test results remained positive after blocking, but with 294 (38%)

the RCs was degraded. In 77(10%) positive responses (RC ≥ 1) prior to blocking, identical RCs were present after blocking. Thirty-five (35, 4.5%) from 38 (5%) negative reactions (RCs= 0) before blocking were negative after blocking, too. Three (3, 0.4%) RCs with negative results (RCs = 0) were enhanced to RC ≥ 1 after blocking and 3 (0.4%) from RC = 3 and RC = 4 were raised to RCs = 5 post blocking (Table 1). The details of modification in RCs to each allergen are shown in tables 1a-1h.

The negative- and positive- predictive values before adding CHO-blocker reagent were calculated 92.1% and 51.2%, respectively (Table 2). Therefore, 92.1% of negative immunologic response before blocking were considered truly negative and 52.1% were regarded as truly positive. According to our results, before blocking, tests indicated high sensitivity 99.2%, while the specificity was low 8.9%.

The absence of immunologic response (RC = 0) after blocking was noticed with varying frequencies for the subsequent allergen extracts: nettle (82%), willow (70%), birch-hazel (65%), mugwort-ragweed (63%), and English plantain (57%), while it was seen much lower in 6-grass mix (29%), rye (22%) and sheep sorrel (20%) (Table 3). Poor congruence of the RC results prior- and post- blocking with all allergens was identified with Cohen‘s kappa (k = 0.045).

Figure 1. The prevalance of polysensitization results of in vitro seasonal allergy tests in dogs from July 2017 to June 2018

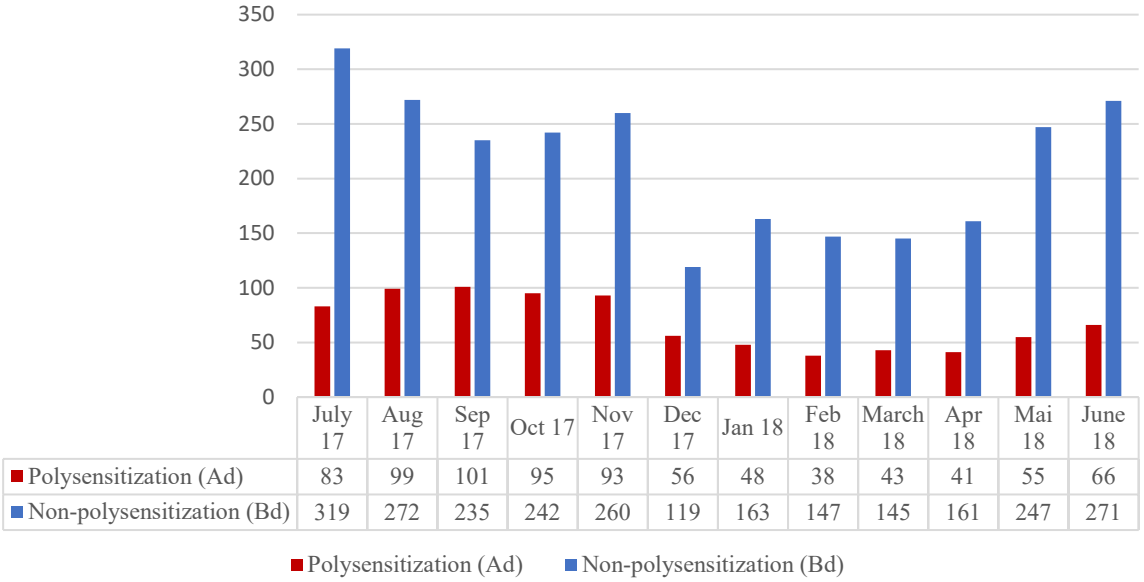

Table 1. Cross table: discrepancy of reaction classes in 96 serum sample results for all tested allergens before- and after CHO blocking in dogs

	RC *	After CHO blocking						
		0	1	2	3	4	5	Total
Before CHO blocking	0	35	2	0	0	0	1	38
	1	55	8	0	0	0	0	63
	2	86	16	11	0	0	0	113
	3	91	26	24	20	0	2	163
	4	49	16	38	15	6	1	125
	5	75	38	48	51	22	32	266
	Total	391	106	121	86	28	36	768

*RC = Reaction class

Table 1a. Cross table of 96 serum sample results with 6-grass mix before- and after-CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	2	0	0	0	0	0	2
	2	6	0	2	0	0	0	8
	3	2	4	2	6	0	0	14
	4	4	4	7	2	1	0	18
	5	14	7	9	12	3	9	54
	Total	28	15	20	20	4	9	96

Table 1b. Cross table of 96 serum sample results with birch + hazel before- and after CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	4	1	0	0	0	0	5
	1	12	0	0	0	0	0	12
	2	20	5	2	0	0	0	27
	3	16	2	4	1	0	1	24
	4	4	2	4	3	0	0	13
	5	6	3	1	3	0	2	15
	Total	62	13	11	7	0	3	96

Table 1c. Cross table of 96 serum sample results with english plantain before- and after- CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	6	1	0	0	0	1	8
	1	3	1	0	0	0	0	4
	2	7	2	0	0	0	0	9
	3	15	6	3	2	0	0	26
	4	12	5	2	1	0	0	20
	5	12	2	7	3	3	2	29
	Total	55	17	12	6	3	3	96

Table 1e. Cross table of 96 serum sample results with nettle before- and after-CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	22	0	0	0	0	0	22
	1	16	2	0	0	0	0	18
	2	20	5	2	0	0	0	27
	3	11	0	0	1	0	0	12
	4	5	0	2	0	1	0	8
	5	5	2	1	0	0	1	9
	Total	79	9	5	1	1	1	96

Table 1f. Cross table of 96 serum sample results with rye before- and after- CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0
	2	3	0	3	0	0	0	6
	3	3	3	2	2	0	0	10
	4	6	1	9	4	2	0	22
	5	9	11	13	11	6	8	58
	Total	21	15	27	17	8	8	96

Table 1g. Cross table of 96 serum sample results with sheep sorrel before- and after- CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO	0	1	0	0	0	0	0	1
	1	1	1	0	0	0	0	2
	2	3	0	0	0	0	0	3
	3	5	2	4		0	0	14
	4	0	2	5	4	1	0	12
	5	9	8	14	16	7	10	64
	Total	19	13	23	23	8	10	96

Table 1d. Cross table of 96 serum sample results with mugwort + ragweed before- and after CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	2	0	0	0	0	0	2
	1	14	2	0	0	0	0	16
	2	8	2	1	0	0	0	11
	3	21	5	4	4	0	0	34
	4	6	2	5	1	1	1	16
	5	9	2	3	2	1	0	17
	Total	60	13	13	7	2	1	96

Table 1h. Cross table of 96 serum sample results with willow before- and after-CHO blocking in dogs

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	7	2	0	0	0	0	9
	2	19	2	1	0	0	0	22
	3	18	4	5	1	0	1	29
	4	12	0	4	0	0	0	16
	5	11	3	0	4	2	0	20
	Total	67	11	10	5	2	1	96

Table 2. Cross Table: impact of CHO blocking and predictive value for all tested allergens in 96 serum samples in dogs

				After blocking		
				Negative	Positive	Total
Before blocking	All allergens		Negative	92.1%	7.9%	100%
			Positive	48.8%	51.2%	100%
		Total		50.9%	49.1%	100%

	Table 3. Cross table: impact of CHO blocking on the predictive value of each allergen in 96 serum samples in dogs					
	Allergens			After blocking		
				negative	positive	Total
Before blocking	6-grass mix		negative	0	0	0
			positive	28 (29)	68 (71)	96
		Total		28 (29)	68 (71)	96
	Birch + Hazel		negative	4 (80)	1	5
			positive	58 (64)	33 (36)	91
		Total		62 (65)	34 (35)	96
	English plantain		negative	6 (75)	2 (25)	8
			positive	49 (56)	39 (44)	88
		Total		55 (57)	41 (43)	96
	Mugwort + Ragweed		negative	2 (100)	0	2
			positive	58 (62)	36 (38)	94
		Total		60 (63)	36 (37)	96
	Nettle		negative	22 (100)	0	22
			positive	57 (77)	17 (23)	74
		Total		79 (82)	17 (18)	96
	Rye		negative	0	0	0
			positive	21 (22)	75 (78)	96
		Total		21 (22)	75 (78)	96
	Sheep sorrel		negative	1 (100)	0	1
			positive	18 (19)	77 (81)	95
		Total		19 (20)	77 (80)	96
	Willow		negative	0	0	0
			positive	67 (70)	29 (30)	96
		Total		67 (70)	29 (30)	96

Values are presented as number of samples (%)

3.2 Results of seasonal allergy tests in cats

A total of 472 feline sera, 222 females (52 intact, 170 spayed), 202 male (40 intact, 162 neutered) and 48 unknowns was investigated. One hundred fifty-five (155) serum samples were excluded due to negative results of allergy tests. For further analyses, 317 samples were included.

One hundred thirty-seven (137, 43%) serum samples were identified as polysensitized (Ac), while 180 (57%) were assigned to the non-polysensitized (Bc) group. The results for polysensitized (Ac) and non-polysensitized (Bc) feline serum samples are shown for each month (Fig 2.) from July 2017 to June 2018.

The minimum prevalence of polysensitization was seen in June with 8 (32%) samples and the maximum in August with 21 (70%) samples. No evidence for marked differences in the polysensitization (Ac) in different seasons was observed. Fourty-eight samples were randomly selected from group Ac. The reaction for each allergen were converted to HERBU (Heska Epsilon Receptor Binding Unit). The HERBU results were converted to 6 classes from 0 to five reaction classes (RC = 0 – 5). From each sample, 8 RCs were shown. From 48 randomly selected samples and 8 allergy panel by each, a total of 384 reaction classes (immunologic reactions) prior- and post- blocking and the impact of CHO reagent in results to all tested allergens is shown in table 4, 5 and 6. The total number of negative responses (RCs = 0) has markedly increased post blocking from 6 (1.56%) to 190 (49%). Thus, from 378 (98%) positive responses (RC $\geq$ 1) before blocking, 184 (48%) RCs were completely inhibited (RCs = 0) after adding CHO reagent solution. One hundred ninety- four (194, 51%) RCs still remained positive after blocking, but showed striking suppression (decreasing the RCs) in 155 (40%) items. Thirty-two percent (32, 8%) of positive responses (RC $\geq$ 1) prior to blocking displayed the same range of RCs post blocking. All six (6, 1.56%) negative responses (RCs = 0) before blocking also stayed negative after blocking. Six (6, 1.56%) RCs of RC = 4 changed to RCs = 5 post blocking (Table 4). The details of

alteration in RCs to each allergen are shown in Table 4a-4h. The negative- and positive-predictive value without adding CHO-blocker reagent were calculated 100% and 51.3%, respectively (Table 5). This means, that all negative results before and after blocking were identical and indicated true negative results. The positive results before blocking remained also positive or true positve in 51.3%. Absence of immunologic response (RC = 0) after blocking was noticed at different percentages for the following allergens: willow and birch-hazel (67%), nettle and mugwort-ragweed (65%), English plantain (54%), in 6-grass mix (31%), sheep sorrel (25%) and rye (23%) (Table 6). No congruence (k = 0,007) was identified prior- and post- blocking within the results of all allergen tests in the randomly selected samples from group Ac.

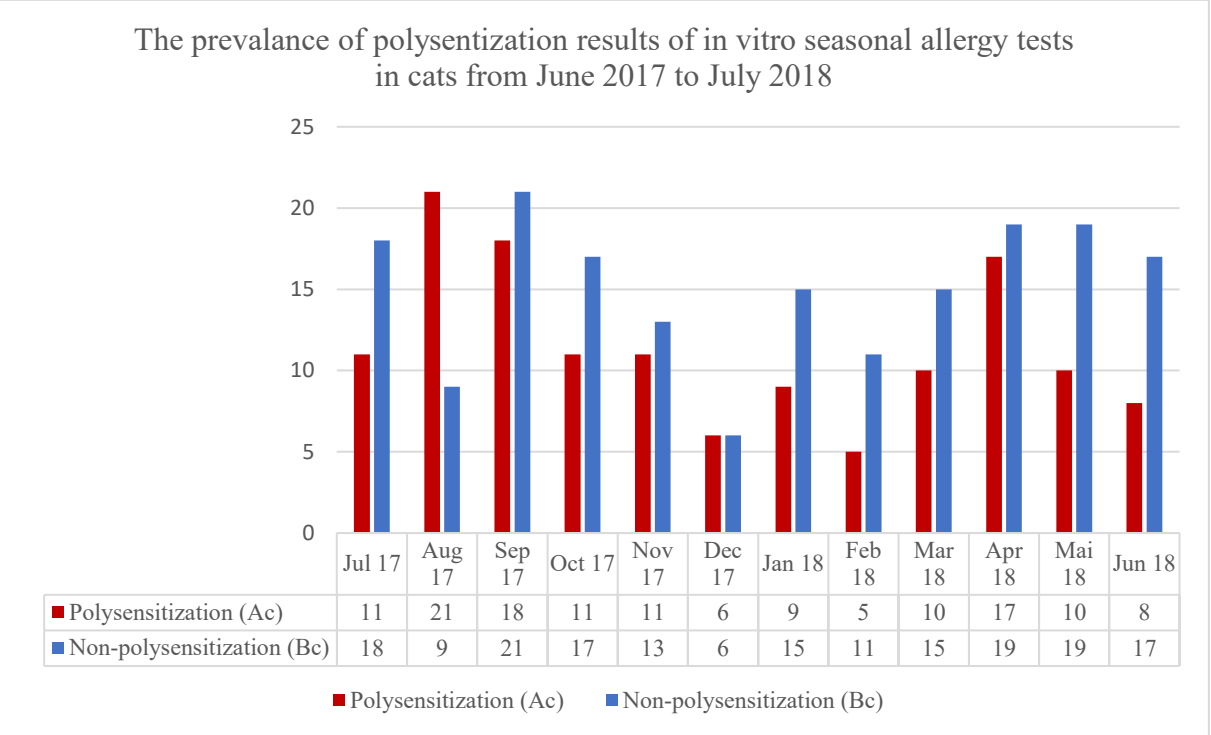

Table 4. Cross table discrepancy of reaction classes in 48 serum samples result to all the allergens before- and after- CHO blocking in cats

	RC *	After CHO blocking						
		0	1	2	3	4	5	Total
Before CHO blocking	0	6	0	0	0	0	0	6
	1	28	1	1	0	0	0	30
	2	54	11	3	0	0	0	68
	3	24	16	13	3	0	0	56
	4	35	10	30	12	4	6	97
	5	43	21	10	22	1 0	21	127
	Total	190	59	57	37	14	27	384

*RC = Reaction classes

Table 4a. Cross table: 48 serum sample results with 6-grass mix before- and after CHO blocking in cats

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	1	1	0	0	0	0	2
	2	3	3	0	0	0	0	6
	3	2	0	3	1	0	0	6
	4	3	0	5	3	1	2	14
	5	6	0	3	4	0	7	20
	Total	15	4	11	8	1	9	48

Table 4b. Cross table: 48 serum sample results with birch + hazel before- and after- CHO blocking in cats

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	2	0	0	0	0	0	2
	1	8	0	1	0	0	0	9
	2	7	1	0	0	0	0	8
	3	6	2	2	0	0	0	10
	4	6	1	1	0	1	1	10
	5	3	2	0	2	1	1	9
	Total	32	6	4	2	2	2	48

Table 4c. Cross table: 48 serum sample results with english plantain before- and after- CHO blocking in cats

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	2	0	0	0	0	0	2
	1	2	0	0	0	0	0	2
	2	8	2	0	0	0	0	10
	3	1	1	3	0	0	0	5
	4	7	2	3	1	0	0	13
	5	6	5	0	3	0	2	16
	Total	26	10	6	4	0	2	48

Table 4e. Cross table: 48 serum sample results with nettle before- and after CHO blocking in cats

	RC *	After CHO blocking						
		0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	10	0	0	0	0	0	10
	2	12	1	0	0	0	0	13
	3	1	2	1	1	0	0	5
	4	3	1	4	2	0	0	10
	5	5	2	0	0	2	1	10
	Total	31	6	5	3	2	1	48

Table 4f. Cross table: 48 serum sample results with Rye before- and after- CHO blocking in cats

	RC *	After CHO blocking						
		0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0
	2	3	1	0	0	0	0	4
	3	1	3	2	0	0	0	6
	4	2	2	7	2	1	1	15
	5	5	5	2	4	2	5	23
	Total	11	11	11	6	3	6	48

Table 4g. Cross table: 48 serum sample results with sheep sorrel before- and after- CHO blocking in cats

	RC *	After CHO blocking						
		0	1	2	3	4	5	Total
Before CHO blocking	0	0	0	0	0	0	0	0
	1	1	0	0	0	0	0	1
	2	2	1	0	0	0	0	3
	3	1	1	1	1	0	0	4
	4	3	2	6	4	1	0	16
	5	5	3	4	5	3	4	24
	Total	12	7	11	10	4	4	48

Table 4d. Cross table: 48 serum sample results with mugwort + ragweed before- and after- CHO blocking in cats

		After CHO blocking						
	RC *	0	1	2	3	4	5	Total
Before CHO blocking	0	1	0	0	0	0	0	1
	1	2	0	0	0	0	0	2
	2	9	1	3	0	0	0	13
	3	7	2	1	0	0	0	10
	4	5	1	2	0	0	1	9
	5	7	3	0	3	0	0	13
	Total	31	7	6	3	0	1	48

Table 4h. Cross table: 48 serum samples result with willow before- and after- CHO blocking in cats

	RC *	After CHO blocking						
		0	1	2	3	4	5	Total
Before CHO blocking	0	1	0	0	0	0	0	1
	1	4	1	0	0	0	0	5
	2	10	3	0	0	0	0	13
	3	5	2	0	0	0	0	7
	4	6	1	2	0	0	1	10
	5	6	1	1	1	2	1	12
	Total	32	8	3	1	2	2	48

Table 5. Cross table: impact of CHO blocking and predictive value for all tested allergens in 48 serum samples in cats

				After blocking		
				negative	positive	Total
Before blocking	All allergens		negative	100%	0	100%
			positive	48,7%	51,3%	100%
		Total		49,5%	50,5%	100%

	Table 6. Cross table: impact of CHO blocking and predictive value to each allergen in 48 serum samples in cats					
	Allergens			After blocking		
				negative	positive	Total
Before blocking	6-Grass mix		negative	0	0	0
			positive	15 (31)	33 (69)	48
		Total		15 (31)	33 (69)	48
	Birch + Hazel		negative	2 (100)	0	2
			positive	30 (65)	16 (35)	46
		Total		32 (67)	16 (33)	48
	English plantain		negative	2 (100)	0	2
			positive	24 (52)	22 (48)	46
		Total		26 (54)	22 (46)	48
	Mugwort + Ragweed		negative	1 (100)	0	1
			positive	30 (64)	17 (36)	47
		Total		31 (65)	17 (35)	48
	Nettle		negative	0	0	0
			positive	31 (65)	17 (35)	48
		Total		31 (65)	17 (35)	48
	Rye		negative	0	0	0
			positive	11 (23)	37 (77)	48
		Total		11 (23)	37 (77)	48
	Sheep sorrel		negative	0	0	0
			positive	12 (25)	36 (75)	48
		Total		12(25)	36 (75)	48
	Willow		negative	1 (100)	0	1
			positive	31 (66)	16 (34)	47
		Total		32 (67)	16 (33)	48

Values are presented as number of samples (%)

4. Discussion

The assumption of the presence of anti-CCD IgE with high cross reactivity was first introduced in 1981 in the *in vitro* allergy test in humans (Aalberse et al. 1981). Anti-CCD IgE neither plays a role in skin prick test in humans nor does in IDST in veterinary medicine (Vidal et al. 2012, Gedon et al. 2019). The phenomenon of anti-CCD IgE leads to strong polysensitization of serological seasonal allergy tests with lack of biologic activity (Altmann 2007). In one study, in 38 CAD (canine atopic dermatitis) patients, individual median power of positive reactions and total power of positive reactions was stronger in sera with the presence of anti-CCD IgE (Levy and DeBoer 2018). In addition, CCDs can also be found in insect venom allergens, helminths and mollusc extracts (Altmann 2007). Despite of phylogenetic relationship mites to insects, N-glycan is not found in house dust mites as they fail to bind rabbit anti-CCD IgE (Wilson et al. 1998). According to one study, no sensitization to mites or moulds was detected in anti-CCD IgE positive human patients (Mari et al. 1999). No discrepancy was seen in the results of mite antigens in IDST and serum test in 31 CAD patients (Gedon et al. 2019). Multi-positive reactions to pollens were reported due to the presence of anti-CCD IgE, though these do not seem to be responsible for the multi-positive reactions to mite extracts (Mari et al. 1999, Gedon et al. 2019). Therefore, CHO-blocker does not seem to influence the immunologic reaction with mite extracts. On the other hand, inhibition of anti- CCD IgE noticeably suppressed the polysensitization results of pollen serum tests, like in humans (Gedon et al. 2019). In our study, 46% of canine and 48% of feline responses of randomly selected samples from the polysensitized groups (Ad, Ac) showed inhibition, or at least suppression 38% and 40% of the reactivity after blocking. Seven (1.82%) immunologic reactions were slightly increased (1 reaction class) in RCs after blocking in cats. In dogs, six (0.8%) immunologic reactions were increased in RCs after blocking. This might be due to the intra-laboratory variability in the second test. In the study by Thom et al., 3.14% of intra-laboratory variability has been identified in allergy testing using Fc-Ɛ receptor test in sera from 15 canine AD (Thom et al. 2010).

Poor congruence with Cohen‘s kappa (k = 0.045) and no congruence (k = 0,007) of the RC results prior- and post- blocking with all allergen extracts were identified in large number of canine and feline samples, respectively. Therefore, the impact of CCD inhibitor in samples from group of polysensitized Ac and Ad was very strong and marked discrepancy was seen in results before and after blocking, as expected based on the Gedon´s study. In the future, evaluation of treatment responses of AIT would be important in order to prove the benefit of this procedure over previously serum allergy testing.

Interestingly, noticeably low percentages of total negative results after blocking were seen in 6-grass mix (29%, 31%), rye (22%, 23%) and sheep sorrel (20%, 25%) in dogs and cats, respectively. These results might indicate true positive reactions in type I hypersensitivity. However, CCD inhibitor may fail to inhibit completely anti-CCD IgE binding to 6-grass mix, rye, sheep sorrel, as well as in other extracts. Further studies with a detailed clinical history are needed.

The role of IgE and correlation with clinical symptoms is still unclear in cats with NFNFIHD. Total negative results were reported up to 35% of cats in a study with IDST and *in vitro* test (Foster and Roosje 2006). In our study, complete negative results were seen in 33% cats before blocking, which is not surprising compared to the previous study (Foster and Roosje 2006). Possibly, these negative results are due to more frequent prescription of glucocorticoids in cats than dogs for two reasons. First, cats can tolerate glucocorticoids better than dogs and second, other anti-pruritic medications such as Oclacitinib (Apoquel®; Zoetis Österreich GmbH, Austria) and Lokivetmab (Cytopoint®, Zoetis Österreich GmbH, Austria) are not licensed for cats with NFNFIHD. There is no study about the presence of anti-CCD IgE in feline sera and comparing with IDST in cats. Further studies on this subject are necessary.

Atopic like dermatitis (ALD) in dogs and human intrinsic atopic dermatitis (IAD) are characterized by absence of allergen-specific IgE against common environmental allergens and cause negative results in allergy tests (Hensel et al. 2015, Kulthanan et al. 2011). The

frequency of ALD was reported between 14% to 25% and that of IAD of 10%-45% (Schmid et al. 2001, Prelaud and Cochet-Faivre 2007, Ott et al. 2009, Kulthanan et al. 2011, Botoni et al. 2019). Although, in our study 26% of dogs have shown total negative reactions prior to adding CHO-blocker, due to the lack or inconsistency of data (like history, e.g. previous medication despite of recommendation to consider withdrawal time prior to the lab testing) this should not be considered ALD.

In conclusion, according to human studies the prevalence of anti-CCD IgE was determined to be more than 20% while two studies in veterinary medicine indicated a prevalence of 24% and 38% in canine sera from AD patients. In our study, the prevalence of polysensitization was 24.07% in sera of dogs and 43.21% in sera of cats, respectively. Seasons of the year did not play a role in the presence of polysensitization in both species. Polysensitization reactions were blocked (46%48%) or suppressed (36%, 40%) with CHO-blocker in dogs and cats, respectively. Rye, 6-grass mix, and sheep sorrel showed higher percentages of positive results after blocking. Finally, performing seasonal allergy *in vitro* test using CCD inhibitor supports the formulation of AIT without interference of non-relevant anti-CCD IgE.

5. Summary

Background – Cross-reactive carbohydrate determinants (CCD) cause polyreactivity in seasonal *in vitro* allergy test. False positive/clinically irrelevant results were identified due to the binding of immunoglobulin IgE against CCD (anti-CCD IgE) with pollen allergens. These were inhibited via adding CCD inhibitor (CHO-blocker) prior to the test.

Objectives - To investigate the prevalence of polysensitized serum samples and evaluate the impact of CCD inhibitor in multi-positive seasonal allergy test results in dogs and cats from July 2017 to June 2018.

Methods and materials – A total of 3399 sera from dogs and 317 cats, submitted for seasonal *in vitro* allergy test via ELISA Fc-Ɛ receptor technology, were studied. Samples were grouped into polysensitized (A) and non-polysensitized (B). Polysensitized samples (A) were retested after adding a modified glycoprotein plant extracts (CCD inhibitor). To determine the impact of CCD inhibitor for each allergen, the reactivity in 96 and 48 randomly selected samples in dogs and cats prior- and post-blocking was investigated.

Results – Polysensitization to seasonal allergens was present in 818 (24.07%) and 137 (43.21%) serum samples of dogs and cats, respectively without depending on seasons. Poor agreement ($\kappa = 0.045$ dogs, $\kappa = 0.007$ cats) of the resultsprior- and post- blocking in 96 (dogs) and 48 (cats) randomly selected samples of group A (d,c) was estimated. CCD inhibitor eliminated (46% dogs, 48% cats) or suppressed (38% dogs, 40% cats) the binding of anti-CCD IgE to allergens. Total negative reactions after blocking were less common in 6-grass mix (29%, 31%), rye (22%, 23%) and sheep sorrel (20%, 25%) in comparison to nettle (82%, 65%), willow (70%, 67%), birch-hazel (65%, 67%), mugwort-ragweed (63%, 65%), and English plantain (57%, 54%) in dogs and cats, respectively. The negative- and positive-predictive value was calculated without blocking (92.1% dogs, 100% cats) and (51.2% dogs, 51.3% cats).

Conclusion – The discrepancy of test results prior to and after blocking was seen in serum samples of polysensitized animals. To improve the quality of seasonal *in vitro* allergy tests, CHO-blocker should be applied in cases suspicious for polysensitized reactions in order to avoid applying not offending allergens in allergen immunotherapy (AIT).

Zusammenfassung

Hintergrund - Kreuzreaktive Kohlenhydrat-Seitenketten (CCD) führen im saisonalen *In-vitro*- Allergietest zu falsch positive durch Seren von polysensibilisierten Tieren. Falsch positive / klinisch irrelevante Ergebnisse aufgrund der Bindung von Immunglobulin IgE an CCD (Anti-CCD IgE) bei Pollenallergenen wurden identifiziert. Diese Bindung wurde vor dem Test durch Zugabe von CCD-Inhibitor (CHO Blocker) eliminiert.

Ziele - Der Prävalenz der Polysensibilisierung in Serumproben und Auswertung der Wirkung von einem CCD-Inhibitor bei multipositiven saisonalen Allergietestergebnissen bei Hunden und Katzen von Juli 2017 bis Juni 2018.

Material und Methoden - Insgesamt wurden 3399 Seren von Hunden und 317 von Katzen, die über die ELISA Fc-Ɛ Rezeptor-Technologie für einen saisonalen *In-vitro*-Allergietest eingeschickt wurden, untersucht. Die Proben wurden in polysensibilisierte (A) und nicht-polysensibilisierte (B) eingeteilt. Seren der positiven Gruppe (A) wurden nach Zugabe eines modifizierten Glykoprotein-Pflanzenextrakts (CCD-Inhibitor) erneut getestet. Um die Auswirkung des CCD-Inhibitors auf ein breites Panel von Allergen zu bestimmen, wurden die Ergebnisse bei 96 und 48 zufällig ausgewählten Proben von Hunden und Katzen nach dem Blocken analysiert und mit den schon vorhandenen Ergebnissen ohne Blocking verglichen.

Ergebnisse - Bei 818 (24,07%) und 137 (43,21%) Serumproben von Hunden bzw. Katzen wurde eine Polysensibilisierung ohne Abhängigkeit von den Jahreszeiten festgestellt. Die Übereinstimmung der Ergebnisse zufällig ausgewählter Proben aus Gruppe A (96 Hunden

und 48 Katzen) vor und nach dem Blocken war schlecht (ϰ = 0,045 Hunde, ϰ = 0,007 Katzen). CHO Blocker konnten die Bindung von Anti-CCD-IgE an Allergene eliminieren (46% Hunde, 48% Katzen) oder unterdrücken (38% Hunde, 40% Katzen). Die Anzahl der insgesamt negativ getesteten Serennach Blocken mit CHO war sehr unterschiedlich und abhängig vom Allergen: 6-Gräser-Mix (29%, 31%), Roggen (22%, 23%) und Sauerampfer (20%, 25%) (82%, 65%), Weide (70%,67%), Birke-Hasel (65%, 67%), Beifuß-Ragweed (63%, 65%) und Spitzwegerich (57%, 54%) bei Hunden und Katzen. Der negative Vorhersagewert ergab ohne Blocken 92.1% (Hunde), 100% (Katzen) und der positive Vorhersagewert 51,2% (Hunde) und 51,3% (Katzen).

Schlussfolgerung - Bei multipositiven Seren wurde eine große Diskrepanz zwischen Testergebnissen vor und nach dem Blocken gesehen. Um die Qualität des saisonalen *In-vitro*-Allergietests zu verbessern, sollte der CHO Block bei multipositiven Ergebnissen angewendet werden, um zu vermeiden, dass durch falsch positive *in vitro* Testergebnisse bei der allergenspezifischen Immuntherapie (AIT) klinisch nicht relevanten Allergene eingesetzt werden.

6. Abbreviations

AD	Atopic dermatitis
AIT	Allergen Immunotherapy
ALD	Atopic like dermatitis
Anti-CCD IgE determinants	Immunoglobulin E against cross-reactive carbohydrate
OD	Optical densities
CCD	Cross-reactive carbohydrate determinants
HD	Hypersensitivity dermatitis
IAD	Intrinsic atopic dermatitis
IDST	Intradermal skin test
IgE	Immunoglobulin E
NFNFIHD	Non-flea, non-food induced hypersensitivity dermatitis FeAD Feline Atopic Dermatitis
A	Polysensitized/ multi-positive results
B	non-polysensitized/ non multi-positive results
RC	Reaction class

7. References

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8. Tables/Figures