The guaiac-based fecal occult blood test (gFOBt) in healthy dogs: evaluation of the diet's effect and the ability to detect fecal occult blood

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Running title: The guaiac-based fecal occult blood tests in dogs

Abstract

Background: The guaiac-based fecal occult blood test (gFOBt) has been used for colorectal cancer screening in humans. It can detect fecal occult blood (FOB) in dogs after oral administration of 20 mg of hemoglobin/kg body weight (mg_{Hgb}/kg_{bw}) of blood and it is influenced by diets.

Objectives: The aims of this work were to evaluate the diet's effect and the ability of gFOBt to detect FOB in healthy dogs.

Methods: Five healthy dogs were fed with HA Purina® and then with EN Purina® diet. Their feces were tested with gFOBt before starting diets and at every defecation during the study period. Every 4 days, increased doses of autologous blood were administered orally. Moreover, whole blood of one dog was progressively diluted with saline solution and dilutions were directly tested with gFOBt, until a negative result was found.

Results: Twelve of 185 (6.5%) gFOBt turned-out positive. No association between blood doses and gFOBt positivity was found. None of the blood-free specimen was positive and 6.5 μg_{Hgb}/mL was the lowest dilution able to achieve all positive tests.

Conclusions: gFOBt was not influenced by both HA and EN diets, but its reproducibility to detect FOB in dogs was unsatisfactory. Individual blood digestion and bowel transit time may play an important role on its scarce reproducibility.

Keywords: bleeding, canine, intestinal, hemoccult, hemoglobin, screening

INTRODUCTION

The guaiac-based fecal occult blood test (gFOBt) has been widely used in human medicine for colorectal cancer screening because of its cheapness and simplicity in implementation.[1,2]

In veterinary medicine gFOBt have been suggested to be useful in patients with chronic hemorrhagic anemias or un-determined enteropathies and in patients receiving prolonged treatment with drugs known to cause gastrointestinal bleedings.[3]

The gFOBt takes the advantage of the pseudo-peroxidase activity of hemoglobin (Hgb): when hydrogen peroxide is added guaiac is oxidized and this reaction induces a subjective color change on the specimen. Positive tests become therefore blue-green.[2]

This kind of test detects the heme moiety and it is not specific neither for human nor for any animal Hgb. Diets containing red meat or having a high peroxidase activity (vegetables like turnips, broccoli, cauliflowers, red radishes, cantaloupes, horseradish, parsnips, cucumbers) can cause false positive results,[4] while diet with high vitamin C content can cause false negative inhibiting peroxidase activity of Hgb.[1]

There are few studies in veterinary medicine about fecal occult blood tests. Two studies evaluated the effect of diet on gFOBt positivity in dogs.[5,6] Using different diets, a poor reproducibility was observed and beef-[6] and mutton-based[5] diets were associated with false positive results. In addition, another study investigated the ability of gFOBt to detect Hgb in stools in six dogs fed with different amount of fresh canine blood: twenty mg of Hgb/kg body weight (mg_{Hgb}/kg_{bw}) was able to cause positive results in all dogs.[7]

The primary aim of this study was to investigate the effect of different diets together with the administration of progressive doses of blood on gFOBt positivity in healthy dogs. Consequently, the effect of time between fecal collection and development of the slides of gFOBt was investigated. Finally, the limit of detection of a gFOBt using progressive doses of canine fresh blood was examined.

MATERIALS AND METHODS

Case selection

Five owned-dogs, from June to December 2017, were prospectively enrolled. Clinical healthy patients of any age, breed and sex were allowed. Data regarding signalment were recorded. This study was authorized by the Ethics and Welfare Council of the University of Pisa (protocol number 0031834/2017). All dogs had no history of chronic diseases and a normal physical examination. Blood samples were also collected, and they were investigated at the Department of Veterinary Sciences, Clinical Pathology Laboratory with complete blood count (Procyte, Idexx Laboratoratories, Milan, Italy) and serum biochemical profile with a biochemistry automated analyzer (Liasys, Assel, Rome, Italy) resulting within the reference range. In addition, dogs were tested with saturated salt fecal flotation and resulted free from fecal parasites.

Tests with diet restriction and administration of progressive doses of whole blood

During the first phase of the study, each dog was fed successively with two different diets. Initially, with an animal protein-free diet, based on hydrolyzed soy (Hypoallergenic HA Purina®, Purina Italia, Italy), then with a diet for gastrointestinal diseases, based on dehydrated poultry proteins (Gastrointestinal EN Purina®, Purina Italia, Italy). No extra foods were allowed, apart from fresh or whey cheeses and dogs did not receive any integration of antioxidants and vitamin C.

At the beginning, each dog was tested for fecal occult blood (FOB) the day before starting the first diet (day 0), then from day 1 each dog was fed with HA Purina® (Figure 1). Starting from day six progressive doses of autologous blood (5, 15, 20, 25 and 40 mg_{Hgb}/kg_{bw}) were orally administered to each dog, every 4 days. Feces were collected at any defecation since day 4 to day 26. Since day 27 up to day 38, dogs have been gradually switched from HA to EN Purina® diet. Considering day 36 as day 1, the same protocol described above was applied to each dog, using EN Purina®. The doses of blood were established based on a previous study that found 20 mg_{Hgb}/kg_{bw} as the minimum dose of blood to achieve 6/6 positive tests.[7] At each time point, the amount of blood (in mL) to be administered was calculated starting from a hypothetical hemoglobin blood concentration of 13 g_{Hgb}/dL using FOR EACH DOG the following formula:

$$\text{Blood volume}, i - d\ (\text{mL}) = \frac{\text{body weight}, i\ (\text{kg}) * \text{ dose of autologous blood}, d\ (\frac{\text{mg}}{\text{kg}})}{130\ (\text{mg/mL})}$$

i, refers to each i-th dog from dog number 1 to dog number 5; *d*, refers to established progressive doses of autologous blood (5, 15, 20, 25 and 40 mg hemoglobin/kg body weight).

Fecal samples were tested with a guaiac paper test (Hemoccult, Beckman Coulter®, Brea, CA, USA) and both storage and analysis were performed according to the manufacturer's instructions.[8] Briefly, each stool specimen was sampled with an applicator stick collecting at least three different stool areas. The specimen was applied to the guaiac paper of the Hemoccult® slide as a thin smear using the provided applicator stick. Slides containing samples were stored at room temperature (15-30°C) until they were developed. Hemoccult® slides were always developed and read by the same operator and results were registered as positive (any blue color appearance within 60 seconds) or negative (no color change). Hemoccult® slides were developed no sooner than 3 days and no later than 14 days after sample application.

Tests without diet restriction

One month after the end of the previous experimental phase, gFOBt were performed on feces of three out of 5 dogs, without diet restriction. Feces were tested once a day for six random days (according to the www.random.org website) in a 12-days interval (30th October – 10th November 2017). The specimen collection, slide storage and developing was done as described above. Moreover, for each fecal specimen, owners had to report type of diet and all extra foods eaten by their dog from the 25th October to the 12th November 2017.

Evaluation of influence of time between sampling and development of the slides

To investigate how the time between fecal collection and development of the gFOBt influenced test results, a single dose of autologous blood (40 mg_{Hgb}/kg_{bw} assuming the hypothetic Hgb blood

concentration of 13 g/dL) was orally administered to dog number 2. Three fecal samples were collected 6, 18 and 42 hours after the blood meal and for each sample collection seven Hemoccult® slides were arranged. Since fecal collection, the slides were developed every two days, from day 2 until day 14, and results were registered as positive (any blue color appearance within 60 seconds) or negative (no color change), as described above.

Evaluation of threshold detection of Hemoccult® test

According to the manufacturer's instructions, Hemoccult® shows 50% of positive tests with 0.3 mg_{Hgb}/g of feces. In this study, another aim was to evaluate laboratory test threshold detection using progressive blood dilutions that were directly applied on the slides. Whole blood (0,5 mL with a hemoglobin concentration of 18 g/dL) was progressively diluted with saline solution and twelve dilutions were obtained: 18 mg/mL, 1.8 mg/mL, 1.0 mg/mL, 0.6 mg/mL, 0.3 mg/mL, 0.1 mg/mL, 50.0 µg/mL, 25.0 µg/mL, 12.5 µg/mL, 7.0 µg/mL, 6.5 µg/mL and 6.25 µg/mL. For each concentration three tests were performed applying directly 100 µL (two drops) of solution on Hemoccult® slides. The developer was added 1-2 minutes after sample application and results were registered as positive when a blue color change was appearing within 60 seconds.

Statistical analysis

At the end of the study, the real administered hemoglobin concentration (rHgb) was calculated starting from the measured hemoglobin concentration (mHgb) for each dog using the following formula:

$$\mathrm{rHgb}, i - d\ \left(\frac{\mathrm{mg}}{\mathrm{kg}}\right) = \frac{\mathrm{mHgb}, i\ \left(\frac{\mathrm{mg}}{\mathrm{mL}}\right) * \text{ blood volume}, d\ (\mathrm{mL})}{\text{body weight}, i\ (\mathrm{kg})}$$

i, refers to each i-th dog from dog number 1 to dog number 5; *d*, refers to established progressive doses of autologous blood (5, 15, 20, 25 and 40 mg hemoglobin/kg body weight);

mHgb, refers to the hemoglobin concentration measured with the complete blood count performed before dogs were enrolled in this study.

Data regarding signalment of dogs, time to develop slides, mHgb and rHgb of each dog, gFOBt positivity related to different diet, doses of autologous blood and a single high dose of blood were analyzed with descriptive statistics. D'Agostino-Pearson test was used to assess the normality of data distribution of times to develop slides and rHgb. Median time to develop slides was compared between positive and negative gFOBt with unpaired Mann-Whitney U test. rHgb and the five expected Hgb doses were compared with unpaired Mann-Whitney U test.

Regarding results obtained during diet restriction, contingency tables were built to evaluate association between positive results and progressive doses of autologous blood as follow: firstly, a 2x2 contingency table was built using all positive and negative results and blood doses divided in <20 mg_{Hgb}/kg_{bw} or ≥ 20 mg_{Hgb}/kg_{bw}. Consequently, a 6x2 contingency table was built using all positive and negative gFOBt and the 6 blood doses (0, 5, 15, 20, 25 and 40 mg_{Hgb}/kg_{bw}). Data were analyzed with McNemar's test and Cochran's Q test, respectively.

Data regarding results obtained with free diet in three dogs and the threshold detection of Hemoccult® test were analyzed with descriptive statistics only.

For statistical analysis, two software (Graphpad Prism 6.0 for Mac OS X, GraphPad Software Inc, La Jolla, CA, USA; SPSS Statistics 25 for Mac OS X, SPSS v. 23, IBM Corp., Armonk, NY, USA) were used and a $P < 0.05$ was considered significant.

RESULTS

Case selection

All the five dogs enrolled were female (1 Border Collie, 1 Cocker Spaniel and 3 mixed-breed dogs). Their age ranged from 2 to10 years old and weight ranged from 11 to 25 kg. A median amount of 0.8 mL (range 0.4-1 mL), 2.5 mL (range 1.3-2.9 mL), 3.4 mL (range 1.7-3.9 mL), 4.2 mL (range

2.1-4.8 mL) and 6.8 mL (range 3.4-7.7 mL) of autologous whole blood was administered to each dog to reach the hypothetical dose of 5, 15, 20, 25 and 40 mg_{Hgb}/kg_{bw}, respectively.

Tests with diet restriction and administration of progressive doses of whole blood

A total of 185 fecal specimens were obtained, 98 with HA Purina® and 87 with EN Purina® diet (Table 1). Twelve of 185 (6.5%) specimens turned out positive, the remaining 173 (93.5%) turned out negative. No dogs were positive for FOB at day 0. Eight (66,7%) of the 12 positive specimens were obtained with HA Purina® diet, 4/12 (33,3%) with EN Purina®. Data are shown in Table 1.

No association between all 6 doses of blood or doses of blood divided in $<20\ mg_{Hgb}/kg_{bw}$ or $\geq 20\ mg_{Hgb}/kg_{bw}$ and positive gFOBt was found.

The median time to develop Hemoccult® slide was 7 days (range 2-17 days). Five of 185 (2.7%) tests were developed beyond 14 days and one of them resulted positive (dog number 4, with EN diet, after 5 mg_{Hgb}/kg_{bw} blood administration). The median time to develop Hemoccult® slides was 9.5 days (range 5-17 days) for positive tests and 7 days (range 2 to 17) for negative tests. No difference between the median time to develop Hemoccult® slides and test outcome was found.

The rHgb was calculated, starting from the administered blood volume, dog weight and mHgb blood concentration for each dog (Table 2). The mHgb ranged from 14.7 to 17.9 g/dL (median 16.7 g/dL). Median rHgb was not significantly higher than expected Hgb dose for each time point (6.7 vs 5 mg_{Hgb}/kg_{bw}, 19.4 vs 15 mg_{Hgb}/kg_{bw}, 26.1 vs 20 mg_{Hgb}/kg_{bw}, 32.1 vs 25 mg_{Hgb}/kg_{bw}, 51.4 vs 40 mg_{Hgb}/kg_{bw}, respectively).

Tests without diet restriction

Dogs number 1, 2 and 3 were enrolled for this part of the study. Eighteen tests (6 tests per dog) were obtained; only one test for each dog (5,5%) was positive. This positivity came from dog

number 2 fed with EN Purina® and that received biscuits, cheese and beef liver as extra-food. Dog number 1 was fed with Royal Canin Light® and received pasta, lamb and beef meat, fruit and cheese as extra food. Dog number 3 was fed with Monge® a commercial diet named "…." and received biscuits, cheese, bread, pizza and beef meat as extra food.

Dog number 1 was fed with Royal Canin Light® and received pasta, lamb and beef meat, fruit and cheese as extra food. Dog number 2 was fed with EN Purina® and that received biscuits, cheese and beef liver as extra-food. Dog number 3 was fed with Monge® a commercial diet named "…." and received biscuits, cheese, bread, pizza and beef meat as extra food.

Evaluation of influence of time between sampling and development of the slides

Hemoccult® slides were developed from 2 to 14 days after sample application, with a median time of 4 days. The only positive test was developed 3 days after sample application. A total of 21 Hemoccult® slides were set, seven for each fecal collection. Only the Hemoccult® slide prepared with the fecal sample collected at 42 hours and developed 12 days after the sample application turned out positive.

Evaluation of threshold detection of Hemoccult® test

From twelve progressive blood dilutions, 33 Hemoccult® slides were obtained. Except for the dilution of 6.25 µg/mL, all the tests were positive. One of the three Hemoccult® slides was negative using the dilution 6.25 µg/mL.

DISCUSSION

The present study has investigated the effect of two different commercial diets and the administration of progressive doses of whole blood on the gFOBt positivity in healthy dogs. gFOBt positivity was not associated with the dose of blood. Although, the amount of 40 mg_{Hgb}/kg_{bw} was

able to produce more positive gFOBt than other doses, the contradictory gFOBt positivity with 5 mg_{Hgb}/kg_{bw} and the absence of positivity with 20 mg_{Hgb}/kg_{bw}, together with the lack of association between gFOBt positivity and escalating dose of blood in the same dog points out a poor reproducibility of gFOBt in dogs.

Few studies have investigated the ability of gFOBt in the detection of occult blood in feces of dogs and cats.[7,9] Six healthy mixed breed dogs were tested for FOB with a gFOBt (Hemoccult II, Smith Kline) after administration of 5, 10 and 20 mg_{Hgb}/kg_{bw} of canine blood and positive results were obtained in two (33%), five (83%) and six (100%) of six dogs respectively.[7] These findings are different if compared with results of the present study. Some reasons can explain differences. In the previous study, all slides were developed daily increasing the risk of false positive due to fecal peroxidase, as previously reported.[7,10] In addition, as a trend toward faster gastrointestinal transit times was observed with increased doses of hemoglobin[7], the advancement to the next higher dose of each dog after two consecutive negative gFOBt, may increase false-positive due to overlap of blood doses.

In the present study, HA and EN diets seemed to have no influence in gFOBt positivity. HA diet was chosen as it is an animal protein-free diet and it should have no influence on Hemoccult® outcome. EN diet was chosen because it is a diet widely used in patients with gastrointestinal diseases and these latter patients are more likely to be tested for occult bleeding. For these reasons, it would have been interesting to evaluate their possible interference with the gFOBt. Interestingly, HA diet gave a bit more positive gFOBt than EN diet (8% and 5%, respectively).

During the second phase of this study, when dogs were fed without diet restriction, one (6%) of 18 fecal specimens resulted positive. The positive specimen was belonging to a dog fed with EN Purina® diet and added with biscuits, cheese and beef liver. This false positive suggests that some ingredients of the diet (e.g. beef liver) can cause false positive in some dogs. In human medicine, recent reviews reported that diet restrictions are not necessary to improve sensitivity and specificity of gFOBt.[4,12] Red meat can cause false positives only assuming large quantities (350-450 g/day)

234 that exceed people's average daily intake of meat.[12] Although, vegetables with high peroxidase
235 activity (turnips, broccoli, cauliflowers, red radishes, cantaloupes, horseradish, parsnips,
236 cucumbers) can cause false positive, as plant-derived peroxidases tend to degrade with time and
237 drying.[4] For this reason current guidelines and test instructions recommend to develop the slides no
238 sooner than two-three days after sample application.[10,12] In dogs and cats, few studies have
239 investigated the influence of diet on gFOBt positivity.[3,5,6] In five healthy dogs fed with seven
240 different diets mutton by-products, mutton liver, beef and brewer's rice were suggested to ~~be able to~~
241 have high peroxidase activity.[5] Another study investigated the influence of nine diets on gFOBt
242 (ColoScreen®, Helena Laboratories) positivity in 6 healthy dogs.[6] Not all diets containing meat
243 gave positive results. The authors speculate a possible influence of dietary fiber to obtain false
244 negative results or different peroxidase activity in the hemoglobin storage or unknown peroxidase
245 or peroxidase-like compounds in the foods.[6]

246 The low rate of positive results in the present study may be due to the difference in test
247 sensitivity between Hemoccult® and other gFOBt, rather than different employed diets and
248 materials and methods. Another reason for different results between studies is the storage conditions
249 of the slides. The first phase of the present study was conducted in the summer period in Tuscany.
250 Storage at room temperature may have influenced results because it was over 30°C associated with
251 high ambient moisture for some days (www.ilmeteo.it), not fully according to manufacturer's
252 instructions. Fecal hemoglobin is not stable and tends to degrade over time. Different seasons and
253 ambient moistures influence Hgb stability and variation in gFOBt positivity is possible.[1,13] In
254 people, gFOBt positivity was demonstrated falling down if slides were exposed to temperatures
255 above 25°C.[14] However, more recent studies have shown that this kind of test works well even with
256 high temperatures (till 45°C).[15] We were not able to find any information regarding this topic in
257 veterinary studies, making this comparison impossible. Even if the second and third phase of the
258 present study were conducted in autumn, no higher rate of positive results were unexpectedly found
259 suggesting a minimal influence of weather conditions as previously reported.[15] However, we would

highlight the importance in mentioning environmental temperature and moisture when a gFOBt is under evaluation to improve result discussions.

In the present study, Hemoccult® was able to detect up to 6,5 μg_{Hgb}/mL of canine fresh blood without false negative results. It is surprising if compared with the low rate of positive results seen in the present study. In human medicine, high doses of ascorbic acid (250 mg daily) in the diet, which inhibits peroxidase activity of hemoglobin, can cause false negative.[16] Both HA and EN vitamin C content (HA doesn't contain vitamin C as additive, EN contains 140 mg/kg of dry food, www.purina.it) is not enough to reach the human threshold and dogs did not receive any ~~integration~~ drugs during the study period. Furthermore, false negatives can arise because stools are not homogeneous, and some portions may have evidence of blood while others do not. This implies that a single slide can turn out negative because the inappropriate portion of the sample was tested.[17] To reduce false negative due to the wrong sampling, fecal specimens were sampled with the applicator stick sampling at least in three different regions. However, it would be advisable to homogenize fecal samples before slide preparation. Moreover, peroxidase activity of Hgb depends on the presence of iron atoms, that can be removed by bacteria during colonic transit.[18-20] We have seen an individual variability in bowel transit time with the different diets. Indeed, different diets tend to modify bowel movements and consistency of the stools in the same dog, suggesting a different digestion and bacterial activity that can influence test results.

This study had some limits. Firstly, the reduced number of enrolled dogs and diets. It is likely that the enrollment of more dogs and the employment of more diets would give more information to discuss. Secondarily, slides were analyzed by the same operator (FB). In a previous study, even if not full, the agreement (86%) between two operators was strong, making this limit minor.[7]

In conclusion, gFOBt seem to be not influenced by both HA and EN diets, and is not useful to detect FOB in dogs. Subjective blood digestion and bowel transit time may play an important role on its scarce reproducibility.

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Table 1: Results of the gFOBt in dogs fed with HA and EN Purina®.

		HA diet **(%)**	EN diet **(%)**	All **(%)**
Before blood	⊕	0 (0%)	0 (0%)	0 (0%)
	⊖	17 (100%)	11 (100%)	28 (100%)
5 mg_{Hgb}/kg_{bw}	⊕	0 (0%)	1 (6.7%)	1 (3.6%)
	⊖	13 (100%)	14 (93.3%)	27 (96.4%)
15 mg_{Hgb}/kg_{bw}	⊕	2 (10.5%)	0 (0%)	2 (6.1%)
	⊖	17 (80.5%)	14 (100%)	31 (93.9%)
20 mg_{Hgb}/kg_{bw}	⊕	0 (0%)	0 (0%)	0 (0%)
	⊖	16 (100%)	14 (100%)	30 (100%)
25 mg_{Hgb}/kg_{bw}	⊕	3 (18.7%)	0 (0%)	3 (8.8%)
	⊖	13 (81.3%)	18 (100%)	31 (91.2%)
40 mg_{Hgb}/kg_{bw}	⊕	3 (17.6%)	3 (20%)	6 (18.7%)
	⊖	14 (82.4%)	12 (80%)	26 (81.3%)

⊕: Positive results; ⊖: Negative results; HA: Hypoallergenic HA Purina®; EN: Gastrointestinal EN Purina®; Before blood: results of tests performed before starting administration of blood; 5-15-20-25-40 (mg_{Hgb}/kg_{bw}): results of tests performed with escalating doses of blood.

Before starting HA Purina® diet, five tests (one each dog) turned out negative.

337

338 Table 2. Data regarding measured and real hemoglobin concentration administered to the five dogs

Dog (n)	**Weight (kg)**	**mHgb (g/dL)**	**Expected Hgb dose (mg_{Hgb}/kg_{bw})**	**Administered blood volume (mL)**	**rHgb (mg_{Hgb}/kg_{bw})**
1	25	16.7	5	1	6.7
			15	2.9	19.4

			20	3.9	26.1
			25	4.8	32.1
			40	7.7	51.4
2	21	14.7	5	0.8	5.6
			15	2.5	17.5
			20	3.4	23.8
			25	4.2	29.4
			40	6.8	47.6
3	12	16.7	5	0.5	7.0
			15	1.4	19.5
			20	1.9	26.4
			25	2.3	32.0
			40	3.7	51.5
4	11	17.9	5	0.4	6.5
			15	1.3	21.1
			20	1.7	27.7
			25	2.1	34.2
			40	3.4	55.3
5	23	17.2	5	0.9	6.7
			15	2.8	20.9
			20	3.7	27.7
			25	4.6	34.4
			40	7.4	55.3

n: number of dogs; mHgb: measured hemoglobin concentration at the beginning of the study; expected Hgb dose: established escalating doses of autologous blood; rHgb: real administered hemoglobin concentration at each dose of blood. Comparison between expected and real Hgb doses were analyzed with unpaired Mann-Whitney U test. A p-value <0.05 was considered significant.

Figure 1. Timeline chart regarding timing of fecal collection and blood administrations. The timeline chart describes timing of fecal collection and blood administrations. Feces were collected before starting the first diet (day 0), at any defecation since day 4 to day 26 and since day 39 to day 61. On day 1 each dog was fed with HA Purina®. Starting from day 6 and day 41 the five progressive doses of autologous blood were administered to each dog, every 4 days. Since day 27 up to day 38, dogs have been gradually switched from HA to EN diet.