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Investigation of interference from canine anti-mouse antibodies in hormone immunoassays

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Abstract

Background: Canine anti-mouse antibodies are a potential source of immunoassay interference, but erroneous immunoassay results are not always easily identifiable. Anti-Müllerian hormone (AMH) is a marker for the presence of gonads in dogs, but elevated AMH concentrations in neutered dogs could also be caused by antibody interference. For other assays, a discrepant result obtained after antibody precipitation might indicate antibody interference.

Objectives: We aimed to evaluate if canine anti-mouse antibodies are a source of erroneous results in the AMH assay and if antibody precipitation with polyethylene glycol (PEG) is a useful tool for detecting antibody interference in a variety of immunoassays used in the veterinary clinical laboratory.

Methods: Twenty-nine positive and 25 negative samples for anti-mouse antibodies were analyzed for AMH, canine total thyroxine (TT_4), canine thyroid-stimulating hormone (TSH), and progesterone before and after treatment with PEG. Results that differed by more than four SDs from the intra-assay coefficients of variation were considered discrepant. Elevated AMH concentrations in neutered dogs with anti-mouse antibodies and no visible gonads present were considered evidence of interference.

Results: Evidence of antibody interference was found in two samples analyzed for AMH. The presence of anti-mouse antibodies did not lead to a higher proportion of discrepant results after PEG treatment for any of the immunoassays. The overall incidence of discrepant results for healthy controls was very high (73%).

Conclusions: Canine anti-mouse antibodies are a source of erroneous AMH results. Antibody precipitation with PEG is not a useful tool for detecting interference caused by such antibodies.

KEYWORDS
antibody, anti-Müllerian hormone, canine, PEG, polyethylene glycol

1 | INTRODUCTION

Since the invention of the radioimmunoassay in the 1950s, immunoassays have become the standard methods for the detection of many clinically important proteins and peptides. Without immunoassays, the diagnosing many medical conditions and being able to monitor these medical conditions at follow-up visits would be seriously affected. However, despite several years of advancements in

immunoassay development, there are still some limitations. One particularly striking flaw inherent to immunoassays is the risk of interference from endogenous antibodies in patient serum and plasma. By emulating the actions of the analyte of interest, these antibodies can cause false-positive results. Immunoassay manufacturers routinely include warnings about interfering "heterophilic antibodies" or "HAMAs" (human anti-mouse antibodies) in package inserts. In practice, this information will usually not reach the veterinary clinician, or could simply be dismissed, perhaps because such antibodies are perceived to be very rare or absent in animals. We have developed a species-independent assay detecting endogenous anti-mouse antibodies and estimated a prevalence of 9% in dogs and 5% in cats.[1]

The suspicion of immunoassay interference could be raised when test results are discordant with the clinical presentation of the patient. An increase marker measurement for no apparent reason could also indicate interference. If the measurement has a high impact on the course of treatment, there is an obvious risk for misdiagnosis and inappropriate therapy. For instance, the anti-Müllerian hormone (AMH) assay is routinely used to determine the presence of gonads in cats and dogs.[2-5] Interference in this assay could lead to unnecessary surgery if patients are incorrectly determined to be unneutered. There have been reports of potentially erroneous AMH results in this assay since it was first evaluated in canine samples,[2,6,7] and antibody interference has previously been described in people.[8]

The presence of discrepant results after various sample treatments is another hallmark of interference. Serial dilutions are sometimes recommended to investigate interference, based on the premise that samples with interfering antibodies will generally display nonlinearity in dilutions. However, the method has been reported to have poor sensitivity with a false-negative rate of 40%,[9] and false-positive results are likely if heterogeneous analytes are being investigated.[10] Blocking of interfering antibodies with nonspecific immunoglobulins (Igs) is another method, but the blocking agents have to be adapted to the specific immunoassay to maximize the chance of success. An alternative approach to tackle immunoassay interference is to deplete the samples of Igs by treating the samples with a precipitant, such as polyethylene glycol (PEG). PEG lowers the solubility of Igs and has been reported to precipitate both serum IgG and IgM efficiently.[11,12] A sample treatment procedure with PEG is quickly performed, and feasible to implement into normal laboratory routines. The present study aimed to evaluate if anti-mouse antibodies detected in a species-independent interference assay are a source of erroneous results in the AMH assay and if PEG treatment is a useful tool for detecting antibody interference in a variety of commercial immunoassays used in the veterinary laboratory.

2 | MATERIALS AND METHODS

2.1 | Samples

Serum that had tested positive for anti-mouse antibodies in a previous screening study were used,[1] no other inclusion criteria were applied. Serum was frozen and stored at −20°C for up to 18 months until analysis. All samples were thawed at room temperature (RT) and thoroughly vortexed before analysis. Exclusion criteria were clearly visible signs of hemolysis, bilirubinemia, or lipemia.

Control sera were collected from the routine laboratory analysis of progesterone at the University Animal Hospital in Uppsala, Sweden, and from a sampling of staff-owned dogs. Serum was frozen and stored at −20°C for up to 3 months until analysis. All samples were thawed at RT and thoroughly vortexed before analysis. Inclusion criteria for control dogs were a negative anti-mouse antibody test [1] and clinically healthy according to medical records and personal communications with owners. Exclusion criteria were clearly visible signs of hemolysis, bilirubinemia, or lipemia.

2.2 | Ethical considerations

The study was approved by the Uppsala Ethical Committee of Animal Experimentation (C 136/13). In accordance with Swedish animal welfare regulations (SJVFS 2015:38), written consent was obtained from all dog owners.

2.3 | Immunoassays

For evaluating the effect of anti-mouse antibodies on AMH concentrations, a sandwich ELISA (AMH Gen II, Beckman Coulter, A79765) was used.

For the PEG screening, a panel of immunoassays was selected for inclusion; AMH Gen II, progesterone, canine thyroid-stimulating hormone (TSH), and canine TT_4 (the last three from Siemens Healthcare Diagnostics). The aim was to include immunoassays that are frequently used in the veterinary laboratory, and/or immunoassays where interference could have a high impact on the course of treatment.

All analyses were carried out on an automated chemiluminescent system (Immulite 2000; Siemens Healthineers) except for the AMH assay, which was performed manually. The AMH and canine TSH assays are both noncompetitive immunoassays. The AMH assay is an ELISA with a pair of monoclonal mouse antibodies; one being used for capture and the other for detection. The TSH assay is a chemiluminescent enzyme immunoassay (CLEIA) with a monoclonal mouse antibody on the solid phase and a polyclonal rabbit antibody as the detection antibody.

The progesterone and canine TT_4 CLEIAs are competitive assays where the sample substances compete with enzyme-labeled analytes for binding to a solid phase capture antibody. In the progesterone assay, the solid phase is coated with a polyclonal rabbit antibody and in the canine TT_4 with a murine monoclonal antibody (mAb). All analyses were performed according to the manufacturers' instructions.

2.4 | Selection of samples and prioritization of the immunoassay order

To evaluate if anti-mouse antibodies are a source of erroneous results in the AMH assay, neutered dogs were used, seven with

anti-mouse antibodies detected using a species-independent assay[1] and seven control dogs. A detectable AMH concentration in neutered dogs with no clinical signs of gonadal tissue present according to patient medical records was considered evidence of interference.

For PEG screening, a general order was established to prioritize when assays were run because of varying sample volumes: (a) AMH; (b) TT_4; (c) TSH; and (d) progesterone. Samples were pretested in the TSH and TT_4 assays to minimize the risk of noninformative results after PEG treatment. Because the TSH results were generally closer to the lower assay detection limit than the TT_4 results, only samples with the 10 highest TSH results were subsequently tested for interference with the PEG method in the TSH assay. The testing of progesterone was limited to intact female dogs. Sample allocation to the different assays is summarized (Table 1). For an overview of all experiments and preparatory steps, see the attached flow chart (Figure 1).

2.5 | Reduction of interference

Two methods were used to reduce interference; blocking with mouse IgG and PEG precipitation.

Blocking with nonimmune mouse IgG was used for neutered dogs with detectable AMH concentrations. Heat-aggregated[13] (MAK33; Roche Molecular Biochemicals, Mannheim, Germany) and/or native mouse IgG (I5381; Sigma Aldrich, St. Louis, MO) was added to the samples in various concentrations. Heat-aggregated MAK33 was kindly provided by N Bolstad (Department of Medical Biochemistry, Oslo University Hospital). After the addition of mouse IgG, the samples were vortexed thoroughly and incubated at 4°C for 30 minutes before analysis. Dilution factors were applied as applicable for different blocking concentrations.

In the screening experiment, PEG precipitation was performed by mixing one volume of serum sample with one volume of 24% PEG-6000 (KEBO Lab, Stockholm, Sweden) reconstituted in 0.01 mol/L PBS, pH 7.4 (Sigma Aldrich). The mixture was vortexed and incubated at 4°C for 30 minutes, followed by centrifugation for 5 minutes at 9600*g* in a Heraeus Fresco 17/21 microcentrifuge (Thermo Fisher, Hemel Hempstead, Hertfordshire, UK). Supernatants were assayed or dispatched immediately to the analyzing laboratory together with the untreated samples, and a 1:2 dilution factor was applied for PEG-treated samples. This treatment was performed on all samples.

When the results from samples treated with PEG and native samples differed by more than four standard deviations (SDs) of the intra-assay coefficient of variation (CV) (Table 2), the result was considered to be discrepant. This equals a 99.99% prediction interval for the difference.

2.6 | Statistical analysis

In the screening experiment with PEG, differences between two proportions were tested using a two-sample test for equality of proportions with continuity correction. For comparisons involving more than two proportions, a generalized linear model was fitted, and a Chi-square test was then performed for H_0:$p1 = p2 = p3$, and so on.

TABLE 1 Assays tested on dogs with anti-mouse antibodies. Descriptive data of sampled dogs and immunoassay allocation of samples investigated for interference

Breed	Age (y)	Sex	Assays tested
Boxer	6	F	AMH, TT_4, TSH, progesterone
Rottweiler	10	F	AMH, TT_4, TSH, progesterone
Poodle	12	M	AMH, TT_4, TSH
Miniature Schnauzer	8	M	AMH, TT_4, TSH
Bernese Mountain Dog	7	MN	AMH, TT_4, TSH
Finnish hound	3	F	AMH, TT_4, progesterone
Schipperke	5	F	AMH, TSH, progesterone
Jack Russell Terrier	4	F	AMH, TT_4, progesterone
Mixed-breed dog	1	M	AMH, TT_4
German Shepherd Dog	4 mo	M	AMH, TT_4
Shetland Sheepdog	11 mo	M	AMH, TT_4
Irish Terrier	7	M	AMH, TT_4
Shetland Sheepdog	9	F	AMH, TT_4
Pug	5 mo	F	AMH, TT_4
American Staffordshire Terrier	7	M	AMH, TT_4
Bearded Collie	7	M	AMH, TT_4
Jagdterrier	9	M	AMH, TSH
Lagotto Romagnolo[a]	5	M	AMH, TSH
Bernese Mountain Dog	8	FN	AMH, TT_4, TSH
Miniature Schnauzer	10	FN	AMH, TSH
German Spaniel	7	F	AMH, progesterone
Papillon	3	MN	AMH
Bernese Mountain Dog	5	FN	AMH
Golden Retriever	11	M	AMH
Mixed-breed dog	1	F	AMH
Mixed-breed dog	9	F	AMH
Chihuahua	13	MN	AMH
English Springer Spaniel	8	FN	AMH

Abbreviations: AMH, anti-Müllerian hormone; CV, coefficient of variation; F, female; M, male; N, neutered; TSH, thyroid-stimulating hormone; TT_4, thyroxine.
[a]Two samples were submitted for this dog.

Pairwise post-hoc estimates were performed using Tukey's range test. All statistical analyses were performed with R Software 3.3.3 (R Core Team, Vienna, Austria).

3 | RESULTS

3.1 | Samples with anti-mouse antibodies

Twenty-nine samples from 28 dogs positive for anti-mouse antibodies were analyzed in at least one of the immunoassays. When submitting two samples from the same dog, 3 days elapsed between the samplings.

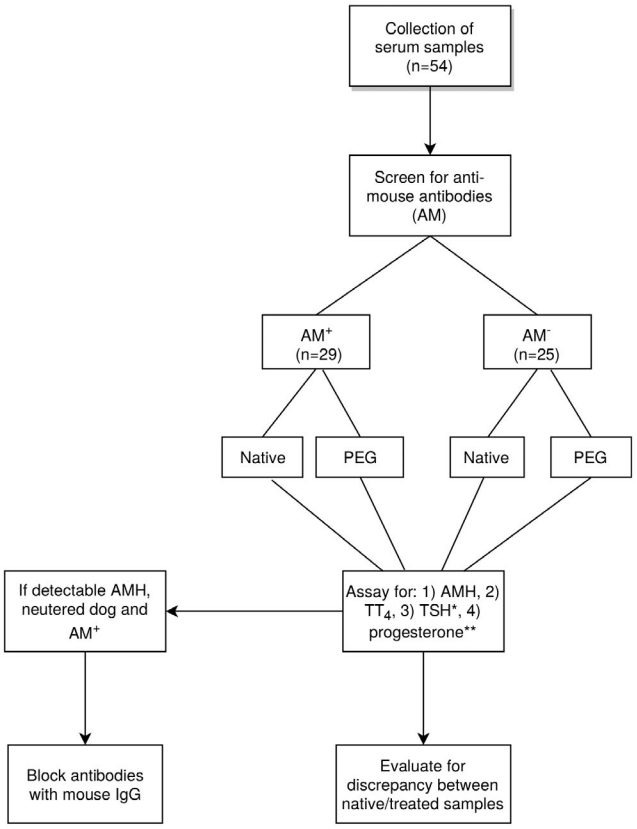

FIGURE 1 A flow chart of the experiments covered in this study. *Only samples with 10 highest TSH measurements (according to pretesting) were evaluated. **Only intact females were evaluated. Abbreviations: AM, anti-mouse antibodies; AMH, anti-Müllerian hormone; PEG, polyethylene glycol; TSH, thyroid-stimulating hormone; TT_4, thyroxine

The median age of the dogs with anti-mouse antibodies was 7 years, IQR 3.75-9 years. There were 11 intact males, 3 neutered males, 10 intact females, and 4 neutered females of 24 different breeds.

3.2 | Control samples

Twenty-five control dogs presenting 22 breeds were included. There were four intact males, five neutered males, 14 intact females, and two neutered females. The median age was 4 years, IQR 2.5- 5 years. Breeds represented included Labrador retriever (n = 3), Berger blanc Suisse (n = 2), and one each of an Australian kelpie, Australian shepherd, Border collie, Border terrier, Cavalier King Charles spaniel, Cesky terrier, English cocker spaniel, flat-coated retriever, German shepherd dog, jack russell terrier, Lancashire heeler, Lhasa apso, malinois, miniature schnauzer, mixed-breed, poodle, Scottish terrier, Sealyham terrier, stabyhoun, and vizsla. All 25 control sera were analyzed in all assays.

3.3 | Effect of anti-mouse antibodies on the result of the AMH assay

Serum from none of the seven neutered control dogs, but serum from two of seven neutered dogs with anti-mouse antibodies yielded detectable AMH concentrations. These two dogs also had the strongest anti-mouse reactivity of the seven neutered dogs when previously screened for interference.

TABLE 2 Immunoassay within-run precision. Within-run CVs of assays investigated for interference. Numbers are only given for the reference ranges that were used in the present study. The in-house CVs were based on duplicate measurements of 11 (TT_4) or 10 (TSH) dogs

Assay	Mean	%CV	$\%CV_{99.99\%}$
AMH (pmol/L)[a]	≤65.9	5.4	21.7
	66	3.6	14.5
Canine TSH (µg/L)	0.08	4.4	20
Canine TT_4 (nmol/L)	32.5	5.7	23.4
Progesterone (nmol/L)[b]	0.92-1.95	8.8	35.3
	1.96-3.23	10.2	40.9
	3.24-7.76	9.7	38.8
	7.77-13.2	7.9	31.7
	13.3-18.1	7	28.1
	≥18.2	7	28

Abbreviations: AMH, anti-Müllerian hormone; CV, coefficient of variation; TSH, thyroid-stimulating hormone; TT_4, thyroxine.
[a]AMH Gen II ELISA Package Insert, 2015;1-6; Beckman Coulter Diagnostics, Brea, CA 92821-6232.
[b]IMMULITE/IMMULITE 2000 Progesterone Package Insert, 2013;1-38; Siemens Healthcare Diagnostics, Tarrytown, NY 10591-5097.

Serum 1 was from a 3-year-old male papillon that according to the medical records had been neutered at another clinic 29 months prior to the collection of serum with no surgical complications. The patient was referred to the University Animal Hospital in Uppsala with acute gastrointestinal signs. A blood test revealed hypoglycemia (2.5 mmol/L, RI 3.8-5.8). After 3 days of intensive care, the patient was released from the hospital, free from clinical signs, and with normalized glucose concentrations.

The initial AMH testing on serum 1 yielded a result of 14.49 pmol/L. Interference testing was performed with concentrations of 0.5 and 1.0 mg/mL heat-aggregated MAK33, 0.5 mg/mL I5381, and a combination of 0.5 mg/mL MAK33 + 0.5 mg/mL I5381 (Figure 2). Heat-aggregated MAK33 (0.5 mg/mL) decreased the result by 22% (to 11.28 pmol/L), and 1.0 mg/mL of heat-aggregated MAK33 decreased the result by 57% (to 6.28 pmol/L). The greatest decrease (62%) was seen with the combination of 0.5 mg/mL heat-aggregated MAK33 + I5381 (to 5.50 pmol/L). The AMH concentrations were below the detection limit when the serum was treated with PEG.

Serum 2 was from a 13-year-old neutered male Chihuahua. The owner sought medical care for the dog after 4 days of postprandial vomiting. After ultrasonographic examination, the primary suspicion was a gallbladder mucocele. Because of the poor prognosis, the dog was euthanized 1 day after admission.

The initial AMH testing on serum 2 yielded a result of 5.71 pmol/L. The same interference testing was performed as for serum 1 (Figure 2). Two treatments yielded undetectable levels of AMH; 1.0 mg/mL heat-aggregated MAK33 and 0.5 mg/mL MAK33 + 0.5 mg/mL I5381.

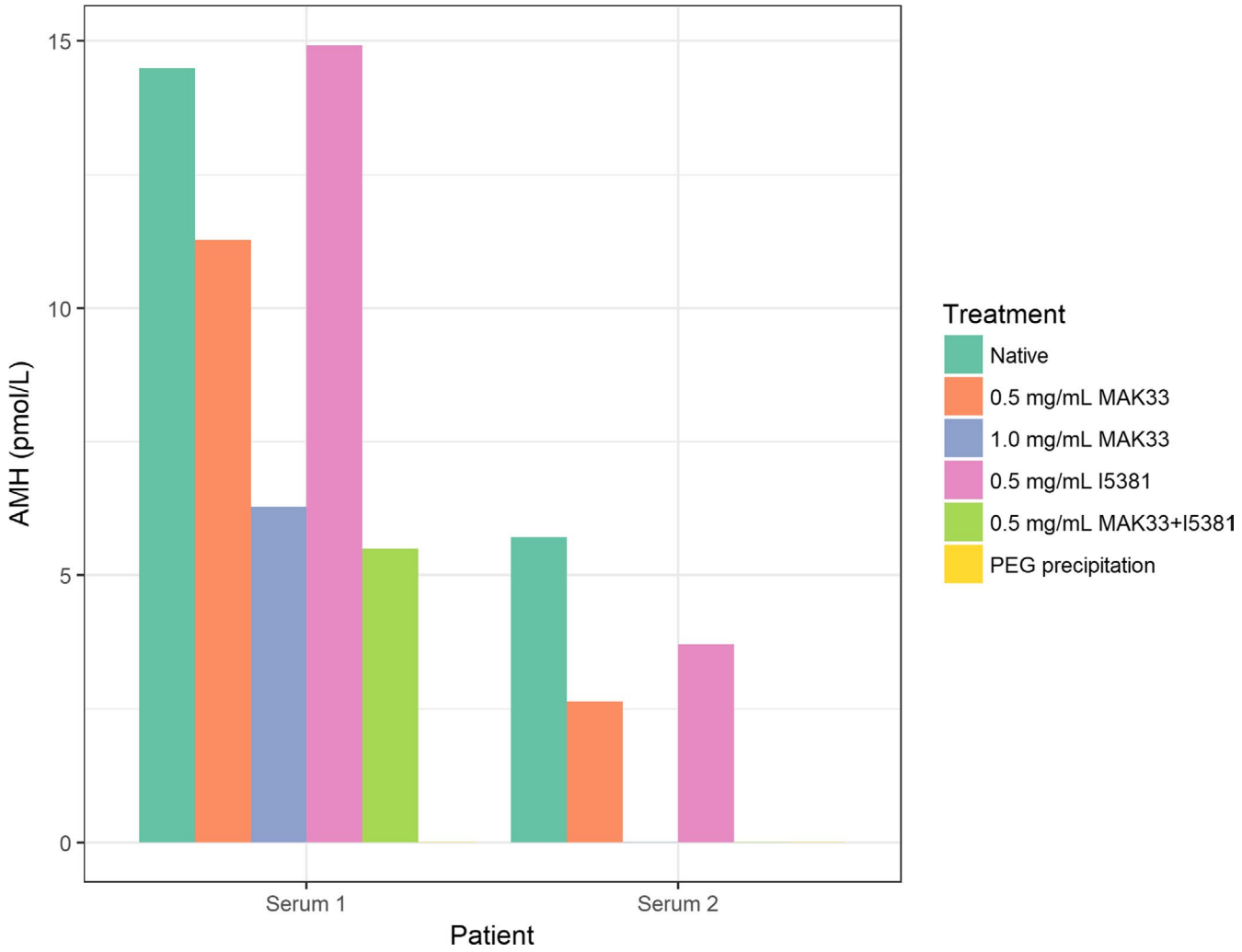

FIGURE 2 Effects of polyethylene glycol (PEG) treatment and antibody blocking on samples with erroneous anti-Müllerian hormone (AMH) results. For blocking, different concentrations of two mouse antibodies (MAK33 and I5381) were used. The antibodies were also used in combination with 0.5 mg/mL of each

Heat-aggregated MAK33 (0.5 mg/mL) decreased the result by 54% (to 2.64 pmol/L) and 0.5 mg/mL I5381 decreased the adult by 35% (to 3.71 pmol/L). The AMH concentrations were below the detection limit when the serum was treated with PEG.

3.4 | Assay screening with PEG

The effects of the PEG treatments are summarized in Table 3. In total, 127 paired analyses were performed, of which 100 (79%) returned informative results.

Fifteen paired analyses (native vs PEG-treated sera) were below the lower detection limit before and after PEG treatment. Three paired analyses were above the upper detection limit before PEG treatment. Nine paired analyses carried out on the Immulite platform returned the error code "NA" after the PEG treatment. Analyses that were not within the assay range before PEG treatment or that returned an error code after PEG treatment were not included in the analyses. When the post-PEG result was below the assay range, the lowest value was divided by two for statistical calculations.

The probability of getting a discrepant result after PEG treatment differed significantly depending on whether AMH, TSH, TT_4, or progesterone was analyzed. This was true for samples with anti-mouse IgG, without anti-mouse IgG, and for all samples ($P < 0.001$ in all three cases). The presence or absence of anti-mouse antibodies did not influence the probability of getting a discrepant result for any of the assays, except the canine TT_4 assay ($P = 0.04$).

Five of the 29 patient samples (17%) with anti-mouse antibodies did not have discrepant results in any of the immunoassays. Conversely, discrepant results were found in at least one of the immunoassays for all 25 serum samples without anti-mouse antibodies.

For assay-specific effects of PEG on samples with anti-mouse antibodies and controls (See Figures 3-6).

4 | DISCUSSION

The present study evaluated if anti-mouse antibodies, detected in a species-independent immunoassay, were a source of erroneous

TABLE 3 A summary of the polyethylene glycol (PEG) effects. The effects on hormone measurements after PEG treatment for samples with (mouse: pos) and without (mouse: neg) anti-mouse antibodies

		AMH	TSH	TT_4	Progesterone	Overall
Number of discrepancies	Mouse: pos	14/14 (100%)	5/10 (50%)	6/14 (43%)	0/6 (0%)	25/44 (57%)
	Mouse: neg	17/17 (100%)	8/10 (80%)	15/18 (83%)	1/11 (9%)	41/56 (73%)
Median percentage of measurement decrease	Mouse: pos	75%	26%	23%	0*	29%
	Mouse: neg	68%	38%	38%	0*	41%

Abbreviations: AMH, anti-Müllerian hormone; CV, coefficient of variation; neg, negative; pos, positive; TSH, thyroid-stimulating hormone; TT_4, thyroxine

*The progesterone measurement increased by 1%.

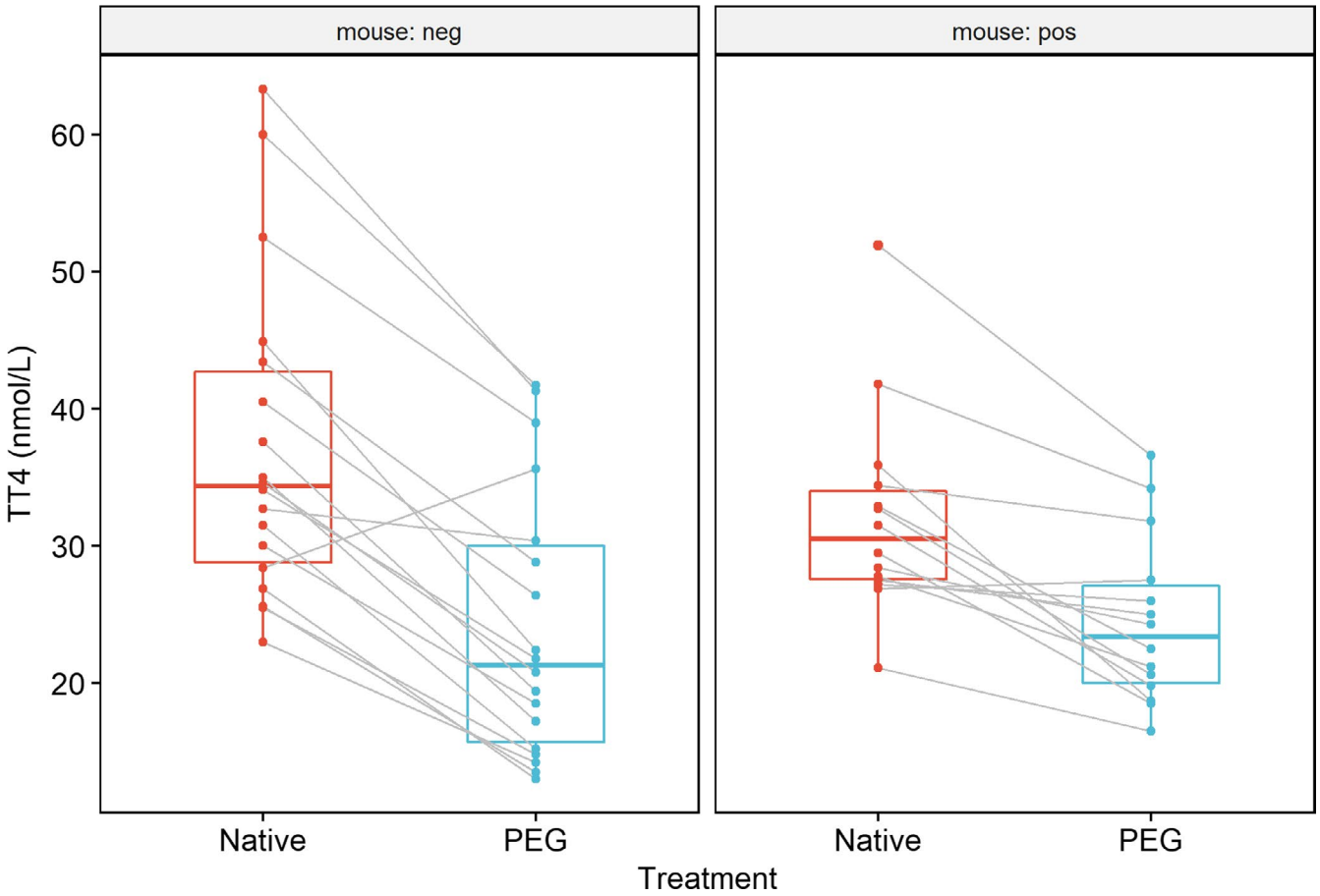

FIGURE 3 The effects of polyethylene glycol (PEG) treatment on canine thyroxine (TT_4) measurements. Healthy control samples negative for anti-mouse IgG are to the left. Patient samples positive for anti-mouse IgG are to the right

results in the AMH assay. We also assessed if antibody precipitation with PEG could be a practical tool for detecting antibody interference in a variety of immunoassays used in the clinical veterinary laboratory.

None of the neutered control dogs but 2/7 neutered dogs with anti-mouse IgG had detectable AMH concentrations. Out of all neutered dogs previously screened for interference, these two dogs also had the strongest reactivity with anti-mouse IgG. Immunoassay manufacturers add neutralizing Igs to their sample incubation buffers, which serve to protect against antibody interference, which is a plausible cause for interference only occurring in samples with the strongest reactivity to mouse IgG. Normal AMH concentrations for neutered dogs of both sexes, as measured with this assay, have been reported to be very low.[2,4,6,14,15] For neutered dogs, this was explained by the absence of Sertoli cells in males, and of granulosa cells in females, which are the only known sources of AMH in mammals[6,16,17] The AMH assay is used for several indications in dogs, including diagnosing the presence of gonads,[2,4-6,14] gonadal tumors,[18,19] and predicting litter size.[20] The interferences found in the present study might not be a big problem for diagnosing tumors, as granulosa and Sertoli cell tumors generally increase AMH concentrations by several magnitudes,[18,19] but they could be misleading when the neutering status of a dog is unknown, such as in cases of suspected ovarian remnants, cryptorchidism, or for stray and

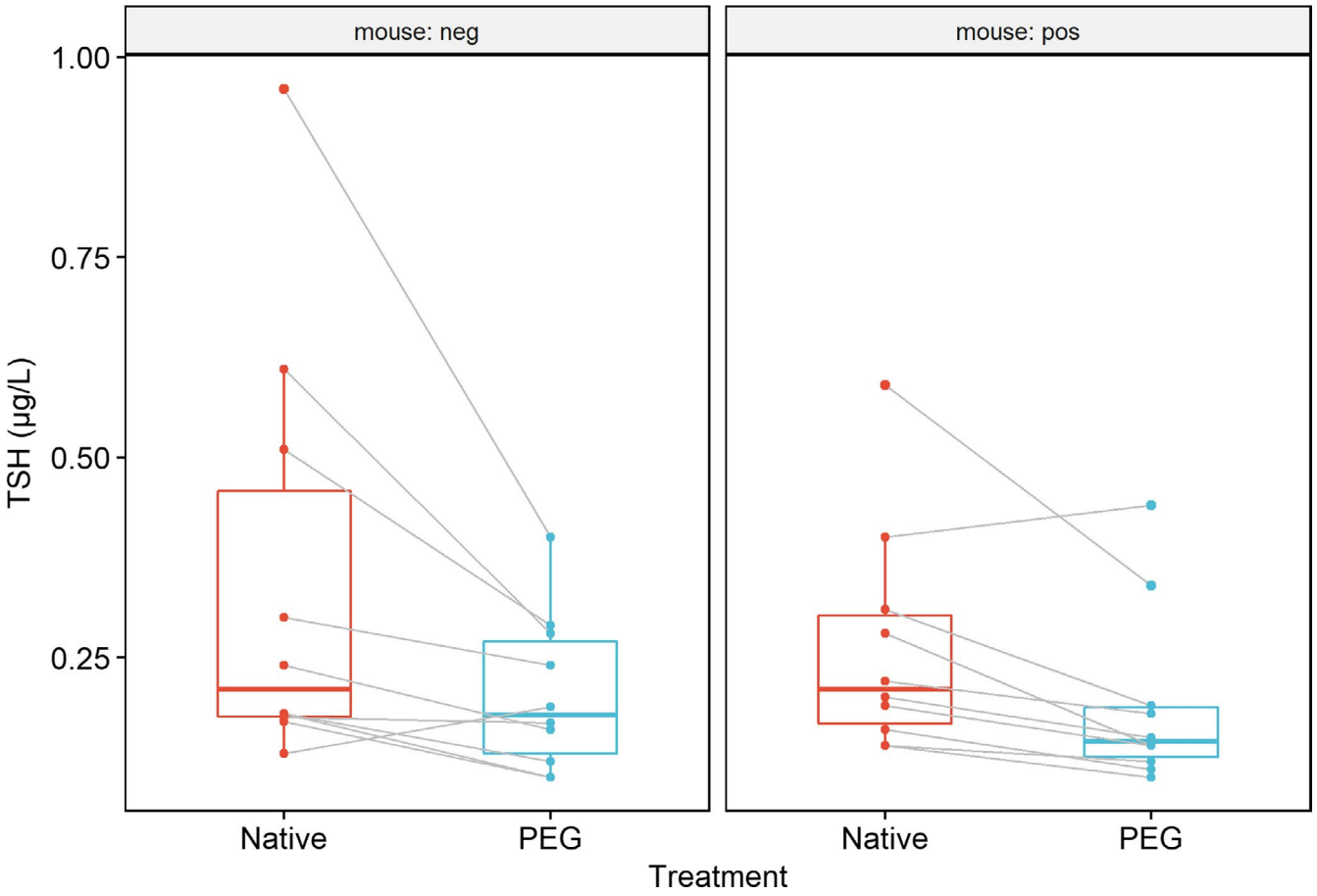

FIGURE 4 The effects of polyethylene glycol (PEG) treatment on canine thyroid-stimulating hormone (TSH) measurements. Healthy control samples negative for anti-mouse IgG are to the left. Patient samples positive for anti-mouse IgG are to the right

surrendered dogs admitted to animal shelters with unknown medical histories. The frequency of interference in canine samples for the AMH Gen II assay is most likely considerably lower than the 9% reported in the screening of canine anti-mouse antibodies since only two of the seven evaluated samples caused erroneous results. This data are consistent with larger cohort (>10 000 samples) studies of human samples submitted for immunoassay analysis, which estimate the frequency of interference to 0.03%-4%.[21-24]

Blocking with two types of mouse IgG was used to reduce the effect of interference. Blocking with 0.5 mg/mL purified mouse polyclonal IgG had little to no effect, but 0.5 mg/mL heat-aggregated MAK33 decreased the AMH concentrations by 22% for serum 1 and 54% for serum 2. Increasing the MAK33 concentration to 1.0 mg/mL decreased the AMH concentrations by 57% for serum 1, and normalized the result from 5.71 to < 0.714 pmol/L in serum 2. Although the mechanism is not fully understood, aggregated antibodies have previously been shown to be superior blockers compared with native IgG.[21] In the same study, it was also shown that 1.0 mg/mL heat-aggregated MAK33 failed to normalize mouse IgG concentrations in only 1 out of 76 human patient samples with heterophilic antibodies. Despite this finding, we interpreted the failure of being able to normalize serum 1 results to be caused by insufficient blocking concentrations because the decrease in titers was proportional to the concentration of MAK33 added. Interestingly, the only other published study assessing blocking of canine heterophilic antibodies showed that 3.7 mg/mL of native IgG was unable to completely eliminate antibody interference,[25] which suggested that higher IgG concentrations might be needed to neutralize interfering antibodies in dogs compared with the corresponding antibodies in people. Both samples displayed linearity after serial dilutions. High AMH concentrations could also be caused by bilateral cryptorchidism, but the explicit mention that patient 1 was neutered ruled out this possibility without reasonable doubt, and it would be difficult to explain the effects after adding MAK33 for either patient in the absence of interfering antibodies. After PEG treatments, AMH concentrations in both sera with erroneous results were below the detection limit, but when used for screening, the proportion of discrepant results did not differ between samples with and without anti-mouse antibodies for any of the immunoassays studied, and a very high overall incidence of discrepant results for healthy controls (73%) was observed. This incidence is unrealistically high. For samples containing anti-mouse antibodies, a 57% incidence of interference might not be out of the question, but the incidence was even higher for samples that did not contain any detectable anti-mouse antibodies. It thus follows that the PEG treatment is responsible for the discrepant results, but in most cases, this is unlikely to be due

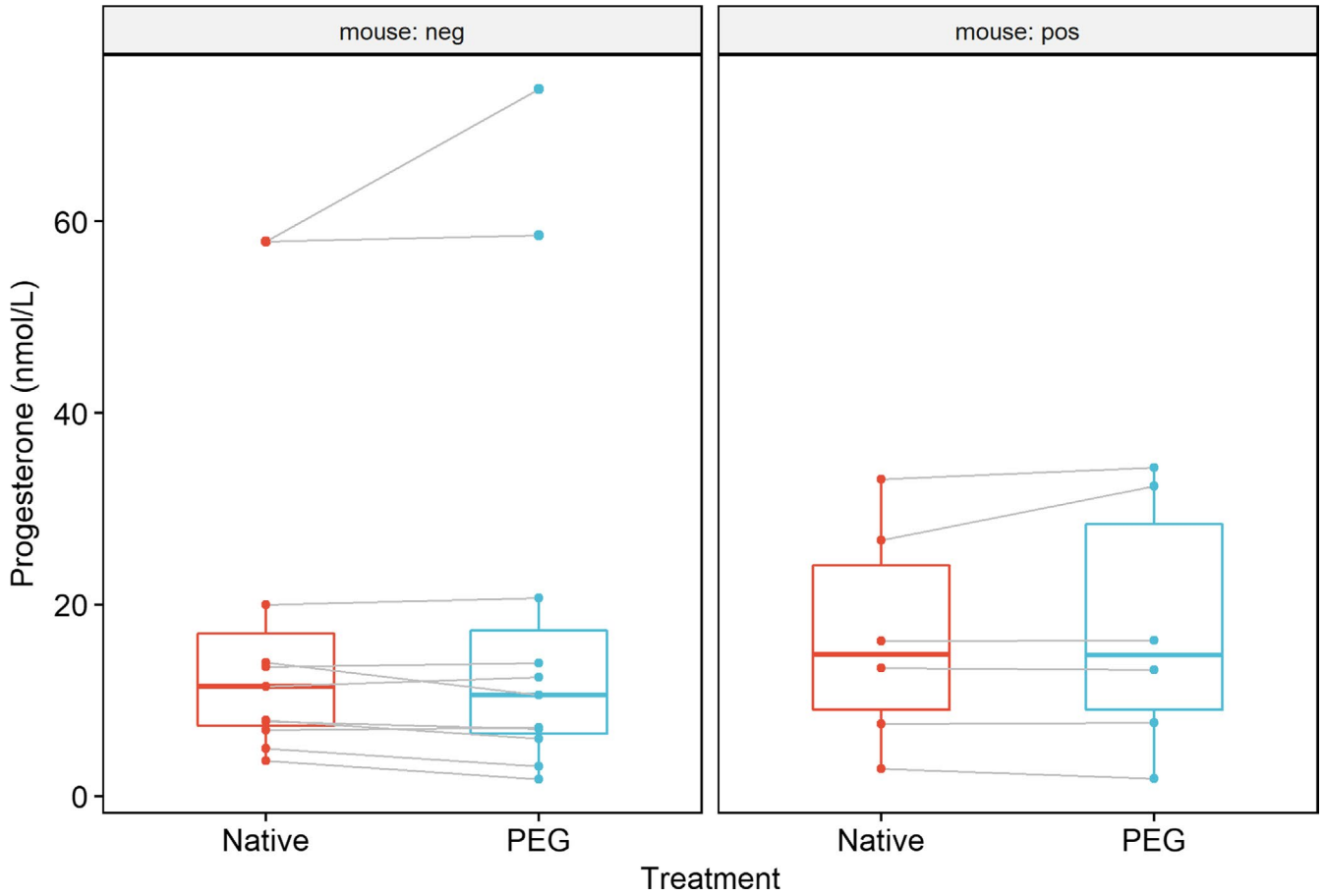

FIGURE 5 The effects of polyethylene glycol (PEG) treatment on progesterone measurements. Healthy control samples negative for anti-mouse IgG are to the left. Patient samples positive for anti-mouse IgG are to the right

to interference. The most likely explanation for the high number of discrepant results obtained with the PEG method is a significant co-precipitation of the analyte with the Igs. A 40% loss of TSH to PEG precipitation has been reported for human samples, but the same study also reported stable values for thyroxine (TT_4).[26] This is in contrast to our findings, where 38% of both TSH and TT_4 were lost in healthy controls. There was also a major apparent co-precipitation of AMH (68% for controls). The progesterone assay seemed to be less affected by co-precipitation, as there was a median increase of 1% in progesterone concentrations for the controls, and only 6% of the analyses yielded discrepant results. Further progesterone assay interference studies could be warranted if the exact concentrations are used to determine the optimal time for mating. Before investigating interference with PEG, laboratories should perform in-house tests for specific analytes on control sera to figure out how big a difference is normally expected after PEG treatment.

An extensive evaluation of methods for removal of heterophilic antibodies in canine plasma was performed by Solter et al.[25] Although the favored protocol was relatively lengthy and involved reagents that may not be standard in laboratories, the preliminary results were encouraging. The methods attempted by the Solter group could be preferable to those attempted by our group in the present study, especially if the method is only to be performed in a few selected cases. We also saw promising results when treating sera that had erroneous AMH results with heat-aggregated IgG. However, this method has to be evaluated more thoroughly before it can be recommended to be used for dog samples. This method is also less convenient to implement in practice, because immunoassays use a variety of different antibodies, and the blocking agent is most effective when it is as similar as possible to the tracer antibody.[27] The fact that antibody interference occurs despite the fact that most (if not all) commercial immunoassay kits are equipped with neutralizing buffers suggests that certain interferences are quite difficult to block with Ig. Blocking solutions tailored to the particular assay are likely to be superior to commercial heterophilic blocking reagents (HBR), which contain multispecies Igs that by chance could be able to bind some interfering substances.[8,28] Furthermore, the addition of IgG is not likely to be effective against interfering antibodies that bind the variable region of the assay antibodies, such as anti-idiotypic antibodies, nor against auto-analyte antibodies.

Although PEG treatment was not useful in detecting canine antibody interference, interference caused by anti-mouse antibodies in the TSH, TT_4, and progesterone assays cannot be excluded. Theoretically, the interference assay is expected to predict interference more accurately for the AMH assay than for any of the other tested assays, because they are both noncompetitive and based on

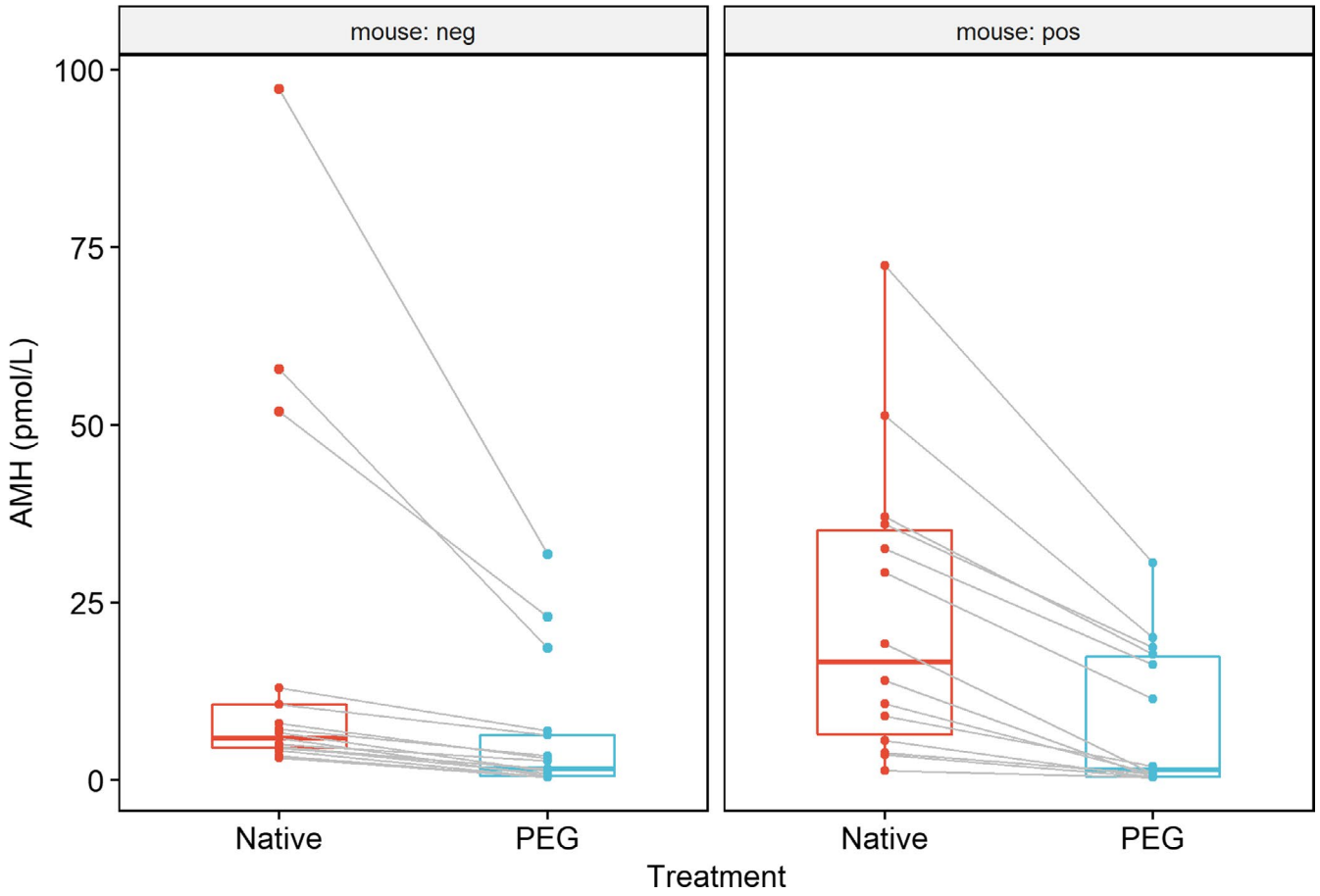

FIGURE 6 The effects of polyethylene glycol (PEG) treatment on anti-Müllerian hormone (AMH) measurements. Healthy control samples were negative for anti-mouse IgG are to the left. Patient samples positive for anti-mouse IgG are to the right

mouse antibodies. The detected antibodies could cause interference in the other assays to a lower extent because most of them use Ig from a species other than mouse for capture and/or detection. Interfering antibodies that bind the constant region of the assay antibodies can react with a multitude of species, including mouse, rabbit, horse, sheep, and bovine IgG,[29,30] but with varying and often low affinities, which reduces the likelihood of interference. The progesterone and TT_4 assays use a competitive format, which is less susceptible to interference than the noncompetitive format,[31] unless the antibodies are of high affinity.[32] High-affinity antibodies can be acquired from mAb therapy, but mAb drugs for veterinary use are not available in Sweden.

When immunoassay interference is discussed, it is often presumed that measurements are falsely increased (positive interference). However, negative antibody interference is also possible, but less commonly observed. With the selection of immunoassays and samples used in this study, we mainly anticipated positive interference. Samples positive for anti-mouse antibodies are defined as such based on their ability to form a bridge between two mouse antibodies (positive interference). If these samples are run in an assay that combines a monoclonal mouse antibody on the solid phase with a detection antibody raised in another species (such as the canine TSH assay), negative interference is possible if only the solid phase antibody is bound.[32] However, noncompetitive assays are run under reagent excess conditions, meaning that the concentrations of the assay antibodies are much higher than normal analyte concentrations,[31] which contributes to a highly sensitive reaction that soaks up any antibody-binding substances in the sample, including the intended analyte. If the anti-mouse antibodies are sufficiently high in concentration and highly specific (ie, iatrogenic HAMAs), the likelihood of saturating the binding sites of the solid phase antibodies and causing negative interference should, in theory, increase. However, such antibodies are not expected to be present in dogs. Competitive assays consisting of a single mouse mAb on the solid phase (such as the canine TT_4 assay) will produce less signal in the presence of anti-mouse antibodies, but because of the inverse relationship between signal and concentration in the competitive format, the reported concentrations will be increased.

Interference can also be caused by cross-reactivity due to structural similarities between the analyte and related molecules. Cross-reactivity is mostly seen in single antibody-assays. Noncompetitive assays that require simultaneous binding of an analyte to two antibodies (such as the AMH assay) have a much higher analytical specificity and are less susceptible to crossreactivity.[31] The manufacturer states that the AMH assay does not detect human inhibin A, activin

A, follicle-stimulating hormone, and luteinizing hormone at two times their physiological concentrations. Equivalent evaluations of cross-reacting canine proteins have not been performed. If cross-reacting substances were the source of the two false-positive results, the concentrations would not be expected to be depressed by the addition of MAK33. Although not definitively ruled out, cross-reactivity is therefore considered to be a much less likely source of interference in the AMH assay.

A limitation of this study is that the addition of PEG entails the risk of introducing dilution effects and volume inaccuracies, which could lead to discrepancies. However, these problems are inherent to this method and to alternative methods such as blocking with the addition of nonimmune antibodies and would still be encountered if the procedure was implemented in a laboratory protocol.

For a laboratory aiming to take proactive measures against antibody interference, it would be favorable that the same protocol is used to identify interference from a variety of antibodies in a variety of immunoassays. According to our results, PEG treatment of canine samples does not seem to provide such a solution. However, it could be a viable option for identifying interference in cases where the analyte is not affected by co-precipitation.

5 | CONCLUSION

Anti-mouse antibodies in dogs are a source of erroneous AMH results. Veterinary clinicians and technicians need to be aware of the risk of immunoassay interference from endogenous antibodies. The PEG method yielded an unrealistically high rate of interference for all examined assays, probably due to co-precipitation of the analyte.

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