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The effect of freeze-thaw cycles on determination of immunoreactive plasma adrenocorticotrophic hormone concentrations in horses

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Abstract

Background: Determination of plasma adrenocotrophic hormone (ACTH) concentration (endogenous or thyrotropin-releasing hormone [TRH] stimulation test) is the most commonly used diagnostic test for pituitary pars intermedia dysfunction (PPID) in horses. Because ACTH is unstable, samples often are frozen to be shipped to laboratories or to allow for batch analysis of research samples. However, the effect of multiple freeze-thaw cycles on equine ACTH is unknown.

Objective: To determine the effects of multiple freeze-thaw cycles on immunoreactive ACTH concentration.

Animals: Twenty-eight horses ranging from 10 to 27 years of age were used.

Methods: Prospective study. Horses were divided into 4 groups: group 1, PPID-negative, without TRH stimulation; group 2, PPID-negative, with TRH stimulation; group 3, PPID-positive, without TRH stimulation; and group 4, PPID-positive, with TRH stimulation. Whole blood was collected from each horse at baseline or 30 minutes after TRH stimulation. Immunoreactive plasma ACTH concentration was determined using a chemiluminescence assay. Plasma samples then were frozen at −80°C >24 hours, thawed at 4°C and reanalyzed for 5 freeze-thaw cycles. Changes in plasma ACTH concentration were analyzed using a linear mixed-effect model.

Results: Significant effects of freeze-thaw cycles (P = .001) and PPID status (P = .04) on plasma ACTH concentration were observed, but no significant effect of TRH stimulation was identified.

Conclusions and Clinical Importance: The plasma ACTH concentration is altered by freeze-thaw cycles, and the effect is observed sooner in horses with PPID. To

Abbreviations: ACTH, adrenocotrophic hormone; CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; POMC, proopiomelanocortin; PPID, pituitary pars intermedia dysfunction; TRH, thyrotropin-releasing hormone; TRHR, thyrotropin-releasing hormone receptor; α-MSH, α-melanocyte stimulating hormone.

diagnose PPID, multiple freeze-thaw cycles should be avoided when measuring plasma ACTH concentration.

KEYWORDS
clinical pathology, diagnostic, endocrinology, pituitary pars intermedia dysfunction, thyrotropin-releasing hormone

1 | INTRODUCTION

Pituitary pars intermedia dysfunction (PPID) is a common endocrinopathy of aged horses and ponies worldwide.[1] Based on previous studies, the prevalence of PPID in horses and ponies >15 years of age is as high as 21%.[1,2] Clinical signs shown in advanced stages of PPID, such as hypertrichosis, pendulous abdomen, or laminitis, can be easily recognized but can be absent in some equids, especially in the early stages of the disease, thus limiting the sensitivity of a clinical diagnosis.[3-5] Currently, necropsy is considered the most accurate method to diagnose PPID. Therefore, it is important to improve antemortem diagnostic testing protocols.[6,7] Determination of plasma adrenocotrophic hormone (ACTH) concentration, at baseline or after thyrotropin-releasing hormone (TRH) stimulation, is the most commonly used test to diagnose or exclude PPID.[5]

Although equine ACTH appears to be more stable than human ACTH, the hormone still is considered to be relatively unstable because of proteolytic degradation.[8-11] In people, blood collection for determination of plasma ACTH concentration is limited to referral centers.[10,11] In other animal species, ACTH has been shown to be somewhat stable after freezing, and therefore preanalytical freezing is common for samples processed in batches for either research or in clinical practice if a delay in sample analysis is expected.[12,13] A previous study suggested that a single freeze-thaw cycle at either −20°C or −80°C had no statistically significant or clinically relevant effect on plasma ACTH concentrations.[8] However, in practice, prolonged transportation at suboptimal temperatures from the site of blood collection to the local laboratory, and ultimately to an endocrine laboratory can result in multiple freeze-thaw cycles. Hence, valid information regarding the effects of multiple freeze-thaw cycles on equine immunoreactive plasma ACTH concentration and the impact on the subsequent diagnosis of PPID is required. Our aim was to identify the effects of multiple freeze-thaw cycles and TRH stimulation on immunoreactive ACTH concentrations in both control horses and horses diagnosed with PPID.

2 | MATERIALS AND METHODS

2.1 | Animals and groups

Twenty-eight adult horses of various breeds, including Standardbred (n = 10), Australian Stock Horse (n = 8), Warmblood (n = 4), Thoroughbred (n = 3), Arabian (n = 2), and Quarter horse (n = 1) were included in the study. There were 13 geldings and 15 mares, ranging from 10 to 27 years of age. Animal usage in this prospective study was approved by the Institutional Animal Care and Use Committee. Horses then were divided into 2 groups of 14 horses, each classified as either PPID-positive or PPID-negative. All horses in non-PPID groups were healthy and all horses in the PPID group had clinical abnormalities only associated with PPID. Horses were individually housed in randomly assigned paddocks at the institution's equine unit where the same feeding and management protocols were applied. A diagnosis of PPID was based on the presence of at least 1 clinical sign (hypertrichosis, polyuria, polydipsia, hyperhidrosis) and an endogenous plasma ACTH concentration >35 pg/mL.[14-17] The study was performed in early summer. In addition to the listed clinical signs, 6 of the 14 PPID-positive horses had hoof changes consistent with chronic laminitis, but none had overt laminitis during the study. Seven horses from each group were randomly selected (coin toss) to receive a TRH stimulation test. Horses were divided into 4 subgroups of 7 horses each: group 1—no PPID using endogenous ACTH; group 2—no PPID using ACTH post-TRH stimulation; group 3—PPID using endogenous ACTH; and group 4—PPID using ACTH post-TRH stimulation.

2.2 | Sample processing

For horses in groups 1 and 3 (without TRH stimulation), whole blood was collected into plastic blood collection tubes containing

TABLE 1 Median, range, and minimum and maximum baseline ACTH concentrations of original unfrozen samples in all groups (in pg/mL)

Group	Median	Range	Minimum	Maximum
1	23.50	16.30	16.50	32.80
2	43.10	22.70	35.30	58.00
3	93.70	76.50	43.50	120.00
4	130.00	244.20	77.80	322.00

Note: Group 1: no PPID using endogenous ACTH, n = 7; group 2: no PPID using ACTH post-TRH stimulation, n = 7; group 3: being PPID using endogenous ACTH, n = 7; and group 4: being PPID using ACTH post-TRH stimulation, n = 7.
Abbreviations: PPID, pituitary pars intermedia dysfunction; TRH, thyrotropin-releasing hormone.

TABLE 2 Estimates of the number of cycles, PPID status, and TRH stimulation status fixed effects using the linear mixed effects model

Variable	Estimates	SE	*P* value
Cycle 0	1.522261	5.823903	.8
Cycle 1	−15.8947	6.640669	.02
Cycle 2	−13.1826	6.317756	.04
Cycle 3	−22.2114	5.286559	<.001
Cycle 4	−12.5041	5.393637	.02
Cycle 5	0	0	-
PPID negative	0.000859	0.00036	.03
PPID positive	0	0	-
Without stimulation	0.000686	0.00036	.07
With stimulation	0	0	-

Abbreviations: PPID, pituitary pars intermedia dysfunction; TRH, thyrotropin-releasing hormone.

ethylenediaminetetraacetic acid (EDTA) after jugular venipuncture for measurement of endogenous plasma ACTH concentration. For horses in groups 2 and 4 (with TRH stimulation), whole blood was collected by jugular venipuncture 30 minutes after the IV injection of 1 mg of TRH (Sigma-Aldrich Pty Ltd [subsidiary of Merck], North Ryde BC, New South Wales, Australia).[18] Within 2 hours of blood sampling, samples were centrifuged at 1000*g* at 4°C for 10 minutes and plasma was separated and placed into cryovials with no additives. After separation, immunoreactive plasma ACTH concentrations were measured using a chemiluminescent assay (Immulite 1000 Chemiluminescent Assay, Siemens, Bayswater, Victoria, Australia). The intra-assay coefficient of variation (CV) for this assay for equine ACTH is reported to be 5.4% and our interassay CV was 4.8%.[19] After analysis, all samples were frozen at −80°C for 24 hours. The samples were thawed at 4°C and reanalyzed immediately after thawing. This process was repeated 5 times. All samples were processed and stored under the same conditions.

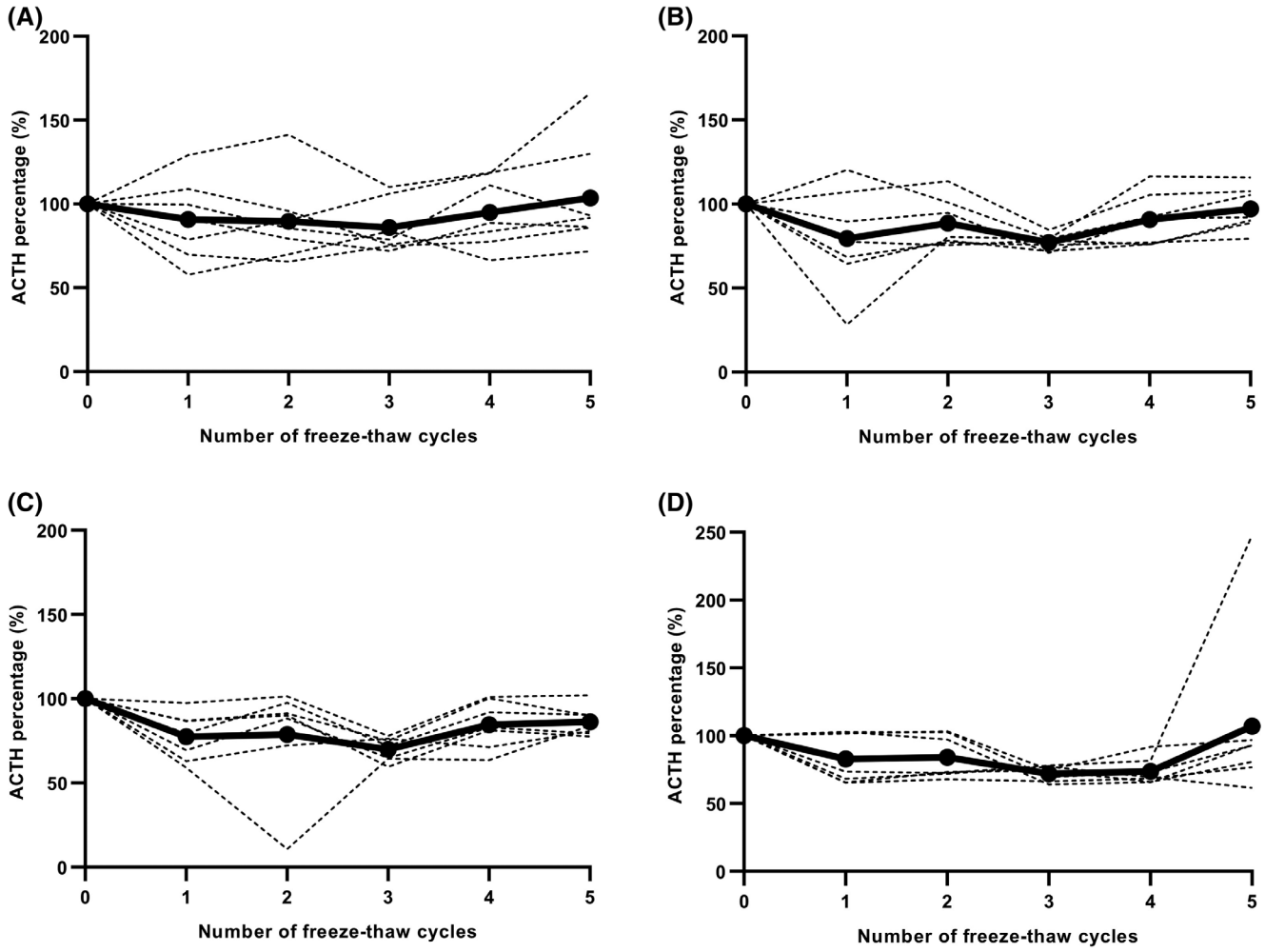

FIGURE 1 Changes in immunoreactive ACTH concentration (as percentage of baseline) over 5 freeze-thaw cycles in group 1 (no PPID using endogenous ACTH, n = 7, panel A), group 2 (no PPID using ACTH post-TRH stimulation, n = 7, panel B), group 3 (being PPID using endogenous ACTH, n = 7, panel C), group 4 (being PPID using ACTH post-TRH stimulation, n = 7, panel D). Data from individual horses are presented by dashed lines and group means are presented by solid lines. PPID, pituitary pars intermedia dysfunction; TRH, thyrotropin-releasing hormone

2.3 | Data analysis

The percentage change in immunoreactive plasma ACTH concentration from the baseline unfrozen sample (Table 1) was calculated for each refrozen sample and satisfied normal distribution as tested using the Kolmogorov-Smirnov test and are presented as mean and SD. Variations in the percentage change of ACTH concentrations then were analyzed by a linear mixed-effect model, with "number of freeze-thaw cycles," "PPID status," and "TRH stimulation" as fixed effects and "horse" as a random effect to account for repeated measures. Because the effect of TRH stimulation did not reach statistical significance, data from groups 1 and 2 and groups 3 and 4 were combined to obtain a control group and a PPID group, respectively, which increased the power of the study ($1 - \beta = 0.65$ overall). Data obtained from control and PPID horses then were compared using a 2-way repeated measures analysis of variance and Tukey's post hoc test. Statistical analysis was performed using commercially available software (IBM SPSS Statistics Version 25 for Windows, Armonk, New York, https://www.ibm.com/products/spss-statistics; GraphPad Prism 8 for Windows 64-bit Version 8.1.2 [332], GraphPad Software Inc, San Diego, California, https://www.graphpad.com) and $P < .05$ was considered statistically significant.

3 | RESULTS

Overall, the number of freeze-thaw cycles and the presence of PPID had a significant effect on immunoreactive plasma ACTH concentrations ($P = .001$ and $P = .03$, respectively) but no significant effect of TRH stimulation was detected ($P = .07$; Table 2). The freezing and thawing process resulted in a significant decrease in immunoreactive plasma ACTH concentration in all 4 groups. On average, compared to unfrozen samples, a 17.4% ± 22.0% decrease in the measured ACTH concentration was observed after the first freeze-thaw cycle, 14.7% ± 22.0% after the second cycle, 23.7% ± 10.8% after the third cycle, 14.0% ± 16.9% after the fourth cycle, and 1.5% ± 35.3% after the fifth cycle (cycle 1, $P = .02$; cycle 2, $P = .04$; cycle 3, $P < .001$; cycle 4, $P = .02$; Table 2). In all groups, immunoreactive plasma ACTH concentration reached a minimum after 3 cycles. This minimum represented a decrease of 85.9% ± 15.6% from the initial concentration in group 1 (Figure 1A), 77.2% ± 4.8% in group 2 (Figure 1B), 70.0% ± 7.1% in group 3 (Figure 1C), and 72.0% ± 5.5% in group 4 (Figure 1D).

When horses were grouped by PPID status (merging groups 1 and 2 and groups 3 and 4; Figure S1), freezing and thawing samples resulted in a significant decrease in immunoreactive plasma ACTH concentration in control horses after 3 cycles ($P = .001$), whereas in PPID horses, a significant effect was detected after the first, third, and fourth cycles ($P = .005$, $P < .001$, and $P = .001$, respectively; Figure 2).

In our study, no erroneous diagnosis or exclusion of PPID occurred after the first freeze-thaw cycle in any group, but 4 PPID cases were missed after 2 freeze-thaw cycles because of the decrease in immunoreactive plasma ACTH concentration with repeated freezing, including 1 case that had a baseline plasma ACTH concentration of 93.7 pg/mL, which decreased to <10.0 pg/mL after 2 cycles. Three equivocal PPID cases were misdiagnosed after 3 freeze-thaw cycles, with immunoreactive plasma ACTH concentrations decreasing from 43.5, 45.6, and 46.6 pg/mL to 33.9, 34.2, and 33.5 pg/mL, respectively. No false-positive diagnoses of PPID occurred after successive freezing and thawing.

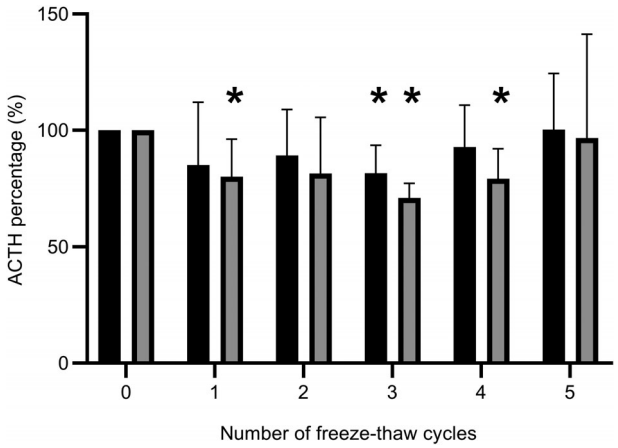

FIGURE 2 Mean and SD of immunoreactive ACTH concentrations (as percentage of baseline) over 5 freeze-thaw cycles in control (n = 14, black columns) and PPID (n = 14, gray columns) groups. *$P < .05$ compared with baseline. PPID, pituitary pars intermedia dysfunction

4 | DISCUSSION

The main results of our study are that (a) the number of freeze-thaw cycles has a significant effect on immunoreactive ACTH concentration; (b) PPID status alters immunoreactive ACTH stability; and (c) TRH stimulation has no significant effect on immunoreactive ACTH stability after successive rounds of freezing and thawing.

Previous research has shown that storage of equine plasma at room temperature impacts immunoreactive plasma ACTH concentration.[20] Our results indicate that multiple freeze-thaw cycles also alter immunoreactive plasma ACTH concentration and that this effect is likely biologically relevant in PPID horses after only 1 cycle. Adrenocorticotropic hormone is a 39 amino acid polypeptide susceptible to proteolytic degradation. Increased temperature during thawing could accelerate the proteolytic process, and therefore the observed decrease could be caused by an enhanced degradation with successive freezing and thawing.[10,21] Interestingly, we also found that, in some samples, immunoreactive plasma ACTH concentration increased after multiple cycles. Similar increases previously were reported after prolonged storage of equine plasma and this increase might be a result of ACTH release from binding proteins altered by multiple freeze-thaw cycles.[20,22] In addition, because the assay originally was designed for use in humans, cross-reactivity with other equine peptide epitopes or cross-species analytical error might have occurred and could have led to the observed individual increases. The changes in immunoreactive plasma ACTH concentrations observed over successive freeze-thaw cycles were higher than the CV

for this assay and appear to be a real finding. Based on our study, no false diagnoses of PPID were identified after 1 freeze-thaw cycle but a statistically significant decrement of plasma ACTH concentration was observed. Therefore, freezing and thawing samples could jeopardize the diagnosis of PPID.

In a study of humans, ACTH was reported to be relatively stable, with 1 or 2 freeze-thaw cycles having limited effect on immunoreactive plasma ACTH concentrations.[23,24] In another study of humans, a significant decrease in plasma ACTH concentration only was observed after 3 freeze-thaw cycles.[25] Although that study used a different assay, our study suggests that in contrast to human ACTH, equine immunoreactive ACTH often decreases after even a single freeze-thaw cycle, especially in horses with PPID. The chemiluminescent assay used in our study is an immunometric assay used in humans that relies on antibodies binding epitopes of the human ACTH peptide (usually directed against amino- and carboxy-terminal regions).[26] Although ACTH is highly conserved among species, equine endogenous ACTH epitopes might be structurally different from those of human ACTH, and only equine ACTH cross-reacting with human ACTH will be measured. Because the epitopes recognized by the assay used in humans are proprietary information, and because the exact structure of various equine proteins cross-reacting and being measured as ACTH is unknown, the observed poor stability of equine ACTH might reflect a lower affinity for assay antibodies or low stability of the specific epitopes of various equine proopiomelanocortin (POMC) degradation products that are being measured as ACTH. Nevertheless, after the third cycle, equine plasma immunoreactive ACTH reached its lowest concentration, which suggests that after 3 freeze-thaw cycles, test results could be extremely unreliable. Therefore, if freezing is unavoidable, limiting it to 2 cycles is recommended, even for horses without PPID.

Plasma samples from horses with PPID were more likely to provide variable and unreliable immunoreactive plasma ACTH concentrations than control horses after multiple freeze-thaw cycles. In control horses, the majority of ACTH originates from the pars distalis of the pituitary gland and only small amounts originate from the pars intermedia of the pituitary gland.[27,28] In PPID, adenomatous hyperplasia of the pars intermedia results in overproduction of POMC-derived peptides.[28-31] Therefore, differences in ACTH stability could be explained by different ACTH origin, and therefore structure, between control and PPID horses. In addition, although ACTH is cleaved into α-melanocyte stimulating hormone (α-MSH) and corticotrophin-like intermediate peptide by prohormone convertase 2 in both healthy and PPID horses, mutation in prohormone convertase 2 in PPID horses decreases conversion to α-MSH and leads to higher plasma concentrations of an ACTH peptide with decreased bioactivity.[32-34] These enzymatic mutations associated with a decreased ACTH bioactivity in PPID horses suggest that the tertiary structure of the ACTH peptide could be different between control and PPID horses, leading to different interactions with the antibodies targeted by the chemiluminescence assay and to more variability in immunoreactive plasma ACTH concentrations. Although no erroneous exclusion of PPID cases occurred in our study after a single freeze-thaw cycle, the statistically significant difference observed in PPID horses between unfrozen and frozen samples could become clinically relevant in a larger population. Therefore, when PPID is suspected, measuring ACTH from an unfrozen sample is recommended.

No differences in plasma ACTH concentrations were detected between basal and post-TRH stimulation samples, which indicates that endogenous ACTH or post-TRH stimulation ACTH might have similar stability after freezing. A larger study with more horses would be needed to confirm this finding. Thyrotropin-releasing hormone receptors (TRHR) are expressed in the pars intermedia both physiologically and pathologically, which leads to TRHR-mediated POMC-derived peptide secretion from the melanotropes in both control and PPID horses.[35] Although horses with PPID respond to TRH stimulation with larger increments in plasma ACTH concentration, TRH only stimulates the release of ACTH stored in the pars intermedia of the pituitary gland.[35] In addition, regardless of PPID status, the production and basal secretion of ACTH from corticotropes in the pars distalis are under a glucocorticoid negative feedback loop, and the POMC-derived peptides from melanotropes in the pars intermedia are entirely under hypothalamic dopamine inhibitory-mediated control.[29,36,37] Therefore, endogenous and post-TRH stimulated ACTH is produced and controlled in the same manner and should respond similarly to freezing and thawing.

Our study had a few limitations. First, the use of a −80°C freezer does not reflect the actual clinical practice situation, in which samples usually are preserved and transported using ice blocks and thermostatic containers. Veterinary practice freezers usually only reach −20°C. Compared with the experimentally controlled freezing and thawing occurring in a controlled laboratory environment as utilized in our study, samples in clinical practice might be exposed to higher and more variable temperatures. Therefore, the effect of freezing and thawing in practice could be more unpredictable than that described here. Our study, however, is very relevant to other research investigators who might freeze samples at −80°C for batch analysis or use archived samples. Another limitation of our study is that only 28 horses were involved, possibly limiting the general value of the results. Although no significant effect of TRH stimulation on equine ACTH stability was detected in our population (Table 2), the *P* value for an effect of TRH stimulation was close to the level of significance utilized (.05) in the study. However, considering that a mix of susceptible ages, breeds, and sex of horses were included, the sample used was deemed adequate to represent a general practice horse population.

To conclude, ACTH concentration is sensitive to the number of freeze-thaw cycles, and PPID status alters immunoreactive ACTH stability. Our results suggest that if PPID is suspected, an unfrozen sample should be used for analysis. For research studies or clinical situations involving non-PPID cases, a maximum of 2 freeze-thaw cycles is recommended. Thyrotropin-releasing hormone stimulation did not appear to alter immunoreactive ACTH stability.

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CONFLICT OF INTEREST DECLARATION

Elizabeth L. Dryburgh is employed by Boehringer-Ingelheim Pty Ltd, and Allison J. Stewart and François-René Bertin have consulted for Boehringer-Ingelheim Pty Ltd.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Institutional Animal Ethics Committee of the School of Veterinary Science, the University of Queensland (SVS/474/17).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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