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ABSTRACT

Cyathostomins are ubiquitous equine nematodes. Infection can result in larval cyathostominosis due to mass larval emergence. Although faecal egg count (FEC) tests provide estimates of egg shedding, these correlate poorly with burden and provide no information on mucosal/luminal larvae. Previous studies describe a serum IgG(T)-based ELISA (CT3) that exhibits utility for detection of mucosal/luminal cyathostomins. Here, this ELISA is optimised/validated for commercial application using sera from horses for which burden data were available. Optimisation included addition of total IgG-based calibrators to provide standard curves for quantification of antigen-specific IgG(T) used to generate a CT3-specific 'serum score' for each horse. Validation dataset results were then used to assess the optimised test's performance and select serum score cut-off values for diagnosis of burdens above 1000, 5000 and 10,000 cyathostomins. The test demonstrated excellent performance (Receiver Operating Characteristic Area Under the Curve values > 0.9) in diagnosing infection, with > 90% sensitivity and > 70% specificity at the selected serum score cut-off values. CT3-specific serum IgG(T) profiles in equines in different settings were assessed to provide information for commercial test use. These studies demonstrated maternal transfer of CT3-specific IgG(T) in colostrum to newborns, levels of which declined before increasing as foals consumed contaminated pasture. Studies in geographically distinct populations demonstrated that the proportion of horses that reported as test positive at a 14.37 CT3 serum score (1000-cyathostomin threshold) was associated with parasite transmission risk. Based on the results, inclusion criteria for commercial use were developed. Logistic regression models were developed to predict probabilities that burdens of individuals are above defined thresholds based on the reported serum score. The models performed at a similar level to the serum score cut-off approach. In conclusion, the CT3 test provides an option for veterinarians to obtain evidence of low cyathostomin burdens that do not require anthelmintic treatment and to support diagnosis of infection.

1. Introduction

Cyathostomins (small strongyles, small redworms) are regarded as the most important parasites of equids. Over the last two decades, anthelmintic resistance has been reported in this group of nematodes with increasing frequency. Benzimidazole and pyrantel resistance, measured as a lack of effectiveness in reducing worm egg shedding at 10–14 days post-treatment, are highly prevalent (Matthews 2014; Nielsen, 2022), with faecal egg count reduction test (FECRT) studies reporting macrocyclic lactone resistance in some regions (Abbas et al., 2021; Bull et al., 2023). In addition to

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the lack of efficacy identified by FECRT, reports of reduced strongyle egg reappearance periods (ERP) after macrocyclic lactone treatment are commonplace (Relf et al., 2014; Tzelos et al., 2019; Nielsen, 2022; Macdonald et al., 2023). Incidences of resistance to the fenbendazole 5-day regime or moxidectin (Reinemeyer et al., 2015; Bellaw et al., 2018; Bull et al., 2023) are particularly concerning. It is clear that all available anthelmintics no longer exhibit levels of efficacy against cyathostomins that were reported when these deworming products were first developed. With, to the best of the authors' knowledge, no new equine anti-parasitics in development, it is important that steps be taken to reduce the further spread of resistance by implementing evidence-based approaches of control (Rendle et al., 2019).

Faecal egg count (FEC) analysis has been strongly promoted to direct anthelmintic treatments to reduce pasture contamination when strongyle egg shedding and nematode transmission are likely to be higher (for example, spring and summer in northern temperate regions). However, there remains a gap in the diagnostic toolbox as FEC tests are incapable of providing accurate information on adult or larval cyathostomin burdens (Dowdall et al., 2002; Nielsen et al., 2010). They provide no information on infection levels in individuals and are of no use in estimating cyathostomin burdens that may, or may not, need targeting by anthelmintics. In particular, cyathostomin encysted larvae can persist for extended periods in the large intestinal mucosa/submucosa and can comprise up to 90% of the total burden (Murphy and Love, 1997; Dowdall et al., 2002). Larval cyathostominosis occurs when these stages emerge simultaneously in large numbers (Giles et al., 1985). Factors associated with this syndrome include the degree of parasite exposure, age, season, concurrent disease and anthelmintic treatment history (Giles et al., 1985; Mair, 1993; Reid et al., 1995; Lawson et al., 2023). Diagnosis is challenging in practice, and historically relies on exclusion of other conditions (Reinemeyer and Herd, 1986).

To address the lack of a diagnostic test that provides information on cyathostomin infection in individuals, an ELISA has been developed based on serum IgG(T) responses to antigens expressed in various developmental stages (Dowdall et al., 2002, 2003, 2004). Following characterisation of IgG(T) responses to larval antigens, recombinant proteins representative of diagnostically informative components were identified by immuno-screening a complementary (c)DNA library using cyathostomin-specific sera (McWilliam, 2010). Genes representing two proteins (Cyathostomin Diagnostic Antigen, CID, and Gut Associated Larval Antigen, GALA) from 14 cyathostomin species were subsequently cloned and expressed (Mitchell et al., 2016; Tzelos et al., 2020). The transcript coding for CID was detected in late mucosal larvae and luminal (larval and adult) stage worms (Tzelos et al., 2020), whilst that coding for GALA was found to be expressed in early L3s (EL3s) and late mucosal larvae (McWilliam et al., 2010). Further studies, examining the relative diagnostic value of the 14 recombinant proteins, identified an antigen cocktail (Cocktail 3; CT3) comprising three proteins from *Cyathostomum catinatum* (protein; Cy-GALA-cat), *Cylicostephanus longibursatus* (protein; Cy-GALA-lon) and *Cylicocyclus nassatus* (protein; Cy-CID-nass), species reported to represent 70–80% of cyathostomin burdens across regions (Bellaw and Nielsen, 2020). Receiver Operator Characteristic (ROC) curve analysis of CT3-specific IgG(T) levels in cyathostomin-infected versus -uninfected horses showed that Area Under the Curve (AUC) values exceeded 0.9, demonstrating that measurement of serum IgG(T) to the CT3 proteins provided excellent performance for detection of infection and for diagnosing mucosal and luminal cyathostomin burdens above thresholds of up to 5,000 worms (Tzelos et al., 2020).

In this study, the CT3 ELISA was subjected to test optimisation and validation in a commercial setting, including the application of total IgG-based calibrators that provide standard curves for quantification of antigen-specific IgG(T), the levels of which are then used to develop a 'serum score' for each tested horse. Also developed, was a high throughput, part-robotic system to facilitate accurate, rapid analysis of multiple samples. The optimised test was first validated using sera from necropsied horses for which cyathostomin counts were available, and the derived ELISA data then used to determine diagnostic 'serum score' cut-off values for determination of burden thresholds above 1000, 5000 and 10000 cyathostomins. The test was then evaluated in populations of live horses grazed in different helminth transmission settings to assess the proportion of each population that would fall above or below a serum score cut-off of 1,000 cyathostomins. Finally, data modelling was undertaken to assess if CT3 serum score results could be used to infer the probability of a horse exceeding different cyathostomin burden thresholds.

2. Materials and methods

2.1. Recombinant antigens used in the CT3 ELISA

Cy-GALA-lon, Cy-GALA-cat and Cy-CID-nass recombinant proteins were expressed and purified as previously described (McWilliam et al., 2010; Mitchell et al., 2016; Tzelos et al., 2020). The GALA and CID proteins were first identified following immuno-screening of a cyathostomin larval (c)DNA library with sera pooled from experimentally infected ponies at 12–16 weeks p.i. (McWilliam et al. 2010). Cy-GALA-cat is derived from a clone identified during the immuno-screening and was ascribed species identity following comparison of its nucleotide sequence with *gala* sequences obtained by PCR amplification from individual identified *C. catinatum* adult worms (Mitchell et al., 2016). For recombinant protein expression, gene-specific primers were used to PCR-amplify *Cy-gala-cat* encoding sequence (minus the signal peptide sequence) from phage plaque eluates. Sequences encoding restriction enzyme sites were incorporated into each primer to facilitate unidirectional cloning into an expression plasmid, pET-22b(+) (Novagen, Merck, Germany). The derived plasmids were transformed into *Escherichia coli* JM109 competent cells (Promega, USA) and selected on ampicillin-Luria-Bertani agar. One colony containing plasmid with an appropriately sized insert was fully sequenced to confirm identity, after which the plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL competent cells (Stratagene, Agilent, USA) for Cy-GALA-cat expression. Cy-GALA-lon encoding sequence was generated using gene-specific primers in reverse transcriptase-PCR amplification of RNA extracted from morphologically identified adult *C. longibursatus* (Mitchell et al., 2016). PCR products were cloned into a pGEM®-T Easy vector (Promega) and the *C. longibursatus gala* sequence confirmed. To generate PCR products for sub-cloning for protein expression, this species-specific sequence was used to design primers incorporating restriction sites to PCR-amplify *gala* DNA from individual identified *C. longibursatus*. The resultant PCR products were digested and ligated into a digested pET-22b(+) vector. Plasmids obtained were confirmed for *gala* sequence and the clones transformed into BL21-CodonPlus(DE3)-RIL cells for expression as above. Similar to Cy-GALA-cat, Cy-CID-nass protein is derived from a cDNA library clone. Nucleotide sequence of the clone was verified as derived from *C. nassatus* by comparing it with *cid* gene sequences obtained by Reverse Transcription (RT)-PCR amplification from individual identified adult *C. nassatus* (Tzelos et al., 2020). The clone was prepared for recombinant protein expression as per Cy-GALA-cat. The three recombinant proteins were expressed and purified as described in Mitchell et al. (2016) and Tzelos et al. (2020), before dialysis with 20 mM sodium phosphate, 0.5 M NaCl (pH 7.4) and storage at −20 °C.

2.2. Development and validation of a commercial optimised CT3 ELISA

Optimisation of the CT3 ELISA for commercial use investigated several factors, including antigen sensitisation (concentration of antigens, antigen combinations, sensitisation buffer), blocking, dilution of serum samples and calibration standards (equine IgG), dilution of conjugate antibody and choice of substrate. For the final optimised assay, wells of 96-well, clear flat-bottom polystyrene high binding microtiter plates (9018, Corning, USA) were coated with CT3 antigen cocktail at 1 μg/ml per antigen (Cy-GALA-cat, Cy-GALA-lon and Cy-CID-nass), diluted in 100 μl sensitisation buffer (0.05 M bicarbonate buffer, pH 9.6) and incubated at 25 °C for 1 h. Dilution of serum samples 1/400 in sample buffer (1x PBS, 0.1% tween 20 and 0.5% BSA) combined with use of a (3,3′,5,5″-tetramethylbenzidine) (TMB) substrate and acidic stop solution, resulted in absorbances within a detectable range at 450 nm. Calibration standards were generated via caprylic acid precipitation of IgG from donor horse serum (defibrinated, UK origin/HA002, TCS Biosciences, UK) as described previously (Lightbody et al., 2016), and prepared by serial dilution. Subsequent to three washes with 200 μl of wash buffer (1x PBS containing 0.1% tween 20), 100 μl of sample buffer (background control), 100 μl of calibration standards (2.5 to 50 μg/ml), and 100 μl of each serum sample, diluted 1/400 in sample buffer, were added in duplicate and incubated at 25 °C for 1 h. Three washes with 200 μl of wash buffer followed, before addition of 100 μl of goat anti-horse IgG(T):Horseradish Peroxidase (HRP) conjugate (A70-105P, Fortis Life Sciences, USA) diluted 1/2,500 or 1/9,000 (dependant on HRP enzymic activity of the conjugate antibody) in sample buffer and a further incubation at 25 °C for 1 h. Following four washes with 200 μl of wash buffer, 100 μl of Invitrogen ELISA TMB Stabilized Chromogen substrate (SB02, Thermo Fisher Scientific, USA) were added and incubated in the dark at 25 °C for 10 min. The reaction was stopped with 100 μl of 1 M hydrochloric acid and absorbance (O.D.) read at 450 nm. For calculation of a 'serum score' for each sample, the mean absorbance reading for each calibrator and sample was determined and the average background control value subtracted. Using the normalised calibrator data, a standard curve (linear model) was produced, and the slope and intercept calculated. A serum score was then calculated using the equation:

$$(Normalised\ sample\ absorbance\ mean - Intercept)/Slope$$

This optimised test protocol was then applied in validation studies using sera from necropsied horses for which cyathostomin burden data were available and in studies assessing CT3-specific IgG(T) in live populations based in geographically distinct regions. A total of 112 serum samples (Supplementary Table S1), including 24 negatives (seven true negatives, 17 presumed negatives), were used for validation of the optimised test. Sera from true negative horses were collected at post-mortem; these were estimated as cyathostomin-free by examination of large intestinal luminal contents and mucosal/sub-mucosal sub-samples using enumeration methods described previously (Dowdall et al., 2002, 2004). Briefly, caecal, ventral colon and dorsal colon contents and mucosal samples were collected separately. Mucosal surfaces were washed to remove luminal parasites, the total volume recorded and a 100 ml sample taken. The total number of nematodes was counted in a 10 ml aliquot and multiplied to give a total luminal burden for each organ. For mucosal counts, the emptied organs were weighed and samples taken from proximal, middle and distal regions to a total weight of 10% of the entire organ. Each sample was divided equally by weight into two; one sub-sample was illuminated from below on a dissecting microscope (transmural illumination) and late L3s (LL3s) and developing L4s (DL4s) counted. Total counts were calculated from the percentage by weight of the sample. The second sub-sample was digested at 37 °C with constant shaking in 1% (w/v) 1:10,000 pepsin/1.5% (v/v) concentrated hydrochloric acid in warm tap water with up to 50 g of tissue in a 200 ml digest solution. Every 30–60 min, digested material was removed by passing it through a 2 mm aperture sieve, and undigested material returned to 200 ml of fresh digest solution. Digestion was continued until no host tissue was retained in the sieve, usually after 3–5.5 h. The digestion material was centrifuged at 450 *g* for 2 min in 250 ml Falcon tubes (Becton Dickinson, UK), the pellet recovered and made up to a volume of 50 ml with 7% (v/v) formaldehyde. Two, 0.5 ml aliquots of each digested sample were examined at 80 × magnification to count EL3s. Total counts were calculated from the percentage by weight of the sample, multiplied to allow for dilution of the pellet.

Due to the high prevalence of cyathostomin infection, negative samples are rare and experimental studies that include necropsy and cyathostomin enumeration are extremely limited. Therefore, true negative samples were supplemented with 17 presumed negative serum samples obtained during two time course studies from foals in France and the USA. Samples used were obtained from foals between 3–7 weeks old. All foals had serum CT3-specific IgG(T) after birth as a result of passive transfer of antibody from mare colostrum. Foals with high CT3-specific IgG(T) maternal antibodies (serum scores >30.0) at 1–4 days old, as measured by the optimised ELISA, were not included due to persistence of maternal antibodies in foal serum. Generally, CT3-specific IgG(T) was observed to decrease over 3–8 weeks, prior to increasing again once foals started mounting a specific IgG(T) response following ingestion of pasture-derived infection. Depending on samples available, sera from time points before or at the start of this increase in CT3-specific IgG(T) were selected for the presumed negative sample cohort; serum scores in these samples ranged from 1.75-14.23 (Supplementary Table S2). The 88 positive sera used were from several populations of infected horses for which cyathostomin burden data were available. A summary of these populations and the associated references for each study are detailed in Supplementary Table S1. All serum samples were stored at −20 °C in small aliquots to minimise freeze/thaw cycles. Most of the validation sample set (110/112) was tested in at least two independent ELISA experiments; however, due to limited sample volume, two samples were tested (in duplicate) in a single experiment.

2.3. Measurement of IgG(T) in naturally infected horses using the optimised CT3 ELISA

CT3-specific IgG(T) was measured in four populations (Supplementary Table S3) using the optimised ELISA. First, serum CT3-specific IgG(T) was measured in a herd of mares and foals (n = 9 pairs) based at the Institute for Agricultural Research (INRA), Nouzilly, France, over 480 days, while the herd was on pasture or stabled. Blood samples were taken from mares and foals at days 0, 1, 2, 3, 7, 14 and 21–25 from the start of the study. From days 30–173, samples were taken at approximate 30-day intervals (range: 23–33 days), from days 173–300, samples were taken at approximate 15-day intervals (range: 12–17 days) and from days 300–480, samples were taken at approximate 31-day intervals (range: 28–34 days). Of these, 23–26 serum samples were tested for each mare and foal pair in this study. The group received occasional anthelmintic treatment (moxidectin, ivermectin or pyrantel embonate, dependent on the individual). Treatment of all foals and most mares with moxidectin (Equest™, Zoetis, USA, dose rate: 400 μ/kg) at stabling enabled assessment of CT3-specific IgG(T) after treatment.

Levels of CT3-specific IgG(T) were also measured in three populations of horses based in Germany (German Yard population; Jürgenschellert et al., 2020, 2022), the UK (Charity Herd) and the

USA (University Herd). In summary, the German Yard population included horses from 47 farms with herds ranging from six to > 100 horses; almost all horses had access to grazing. The majority of farms delivered interval anthelmintic treatments (up to four times per year) in the absence of FEC testing and only four of the farms followed a selective deworming programme, of which three dewormed based on coprological analysis (Jürgenschellert et al., 2020). The Charity Herd comprised an open herd of horses and ponies that came from a range of sources (for example, ex-police and -army horses, welfare relinquishments). This herd has followed a FEC-directed helminth control programme for several years and received annual blanket larvicidal treatments with moxidectin in autumn. Dung removal from paddocks was irregular and not performed on some fields due to topography. The University Herd included horses from the Pioneer 100 Horse Health Project which were donated for various reasons and housed at the Centre for Equine Health at the University of California, Davis, USA. Twelve of the 93 horses tested had access to pasture and the remaining horses were kept on dry lots. CT3 serum scores in these three populations were studied to examine and compare antigen-specific IgG(T) levels in horses across a range of climatic conditions, in a variety of management systems, and with different levels of faecal worm egg shedding. Strongyle FEC data were available for each population, enabling an assessment of how concurrent/recent FEC patterns compared with levels of CT3-specific IgG (T) in serum.

2.4. Statistical analyses

Statistical analyses were performed using Prism version 7.00 (GraphPad Software, USA) and SPSS version 26.0 software (IBM Corporation, USA). CT3 serum scores and TWB (i.e., the total of luminal larval and adult and mucosal larval stage cyathostomins estimated in each animal) threshold levels determined for each of the 112 horses in the validation study were analysed. Three different TWB threshold levels (1,000, 5,000, 10,000 cyathostomins) were chosen to distinguish between positives (those above the TWB threshold) and negatives (those below the TWB threshold). The diagnostic performance of the CT3 serum score to predict cyathostomin TWB levels as positive or negative using these thresholds was determined as sensitivity, specificity, positive and negative likelihood ratios, and positive and negative predictive values, and summarised by ROC curve analysis, including determination of the AUC. Youden index values generated from the ROC curve coordinates were used to identify optimum serum score cut-off values at different cyathostomin burden thresholds. Pearson correlation coefficients were calculated to investigate relationships between serum scores and other variables (for example, age and FEC) for horses in the German Yard group. In cases where significant correlations were found, multivariate logistic regression was used to determine the influence of the variables upon CT3 serum score, using a coding for serum score where scores below the optimum CT3 cut-off = 1, and those above the optimum CT3 cut-off = 0. The serum score selected for this analysis was 14.37 (1,000 TWB; 97.65% sensitivity, 85.19% specificity) and was used to determine the extent to which age and FEC predicted a 'negative serum score'. Frequencies of serum scores $\geq$ 14.37 (positive) or < 14.37 (negative) in equids with recent FEC results (categories compared: positive versus negative FEC; $\geq$200 versus < 200 eggs per gram (EPG)) were compiled for the German Yard group, Charity Herd and University Herd, and compared in 2 $\times$ 2 contingency tables using Fisher's exact test. Serum scores were assessed with respect to nematode transmission risk in populations where there were different levels of strongyle egg shedding between populations using ANOVA with Sidak's multiple comparisons. *P* values of < 0.05 were considered significant in these analyses.

2.5. Development of logistic regression models to convert measured CT3 serum scores to the probability that cyathostomin total worm burden exceeds selected threshold levels

Using SPSS, univariate logistic regression models (Hosmer et al., 2013) were developed to relate CT3 serum scores to the natural logarithm of the odds that an individual would have a TWB above or below a given threshold. The models were based on TWB threshold levels ranging from 0-10,000 mucosal and luminal cyathostomins and serum scores measured for the 112 horses described above. Based on the results of the ROC curve analysis that indicated diagnostic performance was excellent up to10,000 cyathostomins, various TWB thresholds up to this level were assessed (Supplementary Table S4). The models contained the following terms:

$$\text{Logit} = \text{constant} + (\text{coefficient x serum score})$$

where:

logit = natural logarithm of the odds that TWB exceeds the threshold

The relationship between logit and probability can be expressed as follows:

$$\begin{aligned}\text{logit} &= \ln(\text{odds TWB exceeds threshold}) \\ &= \ln(p/(1-p)) \text{ where } p \\ &= \text{probability that TWB exceeds threshold}\end{aligned}$$

Applying the models to the serum score measured for each horse produced logit values. These were converted to the probabilities that the TWB exceeded the given threshold levels.

3. Results

3.1. Validation of the optimised CT3 ELISA in a commercial setting using serum from horses with enumerated cyathostomin burdens

Analysis of the CT3 ELISA validation dataset demonstrated high ROC-AUC values for cut-off thresholds up to, and including, 10,000 mucosal and luminal cyathostomins (Supplementary Fig. S1; Table 1; ROC-AUC range 0.910–0.956). At higher cyathostomin thresholds, ROC-AUC values were less than 0.9, the level accepted as excellent diagnostic utility (Swets, 1988). To investigate serum score thresholds to use in the test's application in equine practice, values up to serum score 50.0 were assessed from the ROC curve coordinates by examining the trade-off of diagnostic sensitivity against specificity. Serum scores in the maximal zone of the Youden index (i.e., J = sensitivity + specificity – 1) were interrogated and values selected in this region for TWB threshold cut-offs of 1,000 mucosal/luminal cyathostomins (serum score cut-off: 14.37), 5,000 mucosal/luminal cyathostomins (serum score cut-off: 15.61) and 10,000 mucosal/luminal cyathostomins (serum score cut-off: 30.46). These serum score cut-offs provided sensitivity of 91.55–97.56% and specificity of 71.43–85.19%, depending on the selected TWB threshold. Positive predictive values ranged from 86.67-95.40% and negative predictive values, 83.78–92.00%, with highest values calculated at the 1,000 TWB cut-off (serum score 14.37; Table 2).

3.2. Measurement of CT3-specific IgG(T) in mares and foals over 480 days

To provide information on the dynamics of serum IgG(T) in naturally infected populations subjected to anthelmintic treatment and to provide data to inform timing of CT3 ELISA testing post-treatment, a time course study was undertaken in a mare and foal population based at INRA. CT3 serum scores obtained for these mares and foals over time are depicted in Supplementary Fig. S2.

Table 1
Receiver Operating Characteristic-Area Under the Curve (ROC-AUC) analysis of CT3-specific IgG(T) in cyathostomin-infected horses stratified according to different total worm (cyathostomin) burden (TWB) thresholds. The samples analysed (n = 112) included sera from 24 equids classified as negative (seven true negatives, 17 presumed negatives) and 88 infected equids from different studies for which cyathostomin mucosal and luminal cyathostomin burdens were available (Supplementary Table S1). Equids were categorised as below or above the following TWB thresholds: 1,000, 5,000, and 10,000 mucosal and luminal cyathostomins, and the number of horses above and below each threshold are indicated. The ROC-AUC values and 95% confidence intervals (CI) obtained at the three cyathostomin TWB threshold cut-off levels are shown.

TWB threshold (mucosal and luminal cyathostomins)	Horses below TWB threshold	Horses above TWB threshold	ROC-AUC values	95% CI
>1,000	27	85	0.956	0.914–0.998
>5,000	35	77	0.919	0.868–0.971
>10,000	41	71	0.910	0.852–0.969

Table 2
CT3 serum score thresholds selected using values from the maximum Youden index zone for a range of total worm burden (TWB) thresholds (1,000, 5,000, and 10,000 mucosal and luminal cyathostomins). The proportion of horses classified as negative and positive at each TWB threshold is shown in Table 1. Sensitivity and specificity values were calculated from Receiver Operating Characteristic-Area Under the Curve (ROC-AUC) analysis using the 112 validation samples (Supplementary Table S1) assessed in the optimised CT3 ELISA. Positive and negative likelihood ratios and positive and negative predictive values are shown for each TWB threshold. The 95% confidence intervals (CI) for each parameter are included.

TWB threshold[a,b]	Serum score threshold	Sensitivity (95% CI)	Specificity (95% CI)	Positive Likelihood Ratio (95% CI)	Negative Likelihood Ratio (95% CI)	Positive Predictive Value (95% CI)	Negative Predictive Value (95% CI)
>1,000	14.37	97.65% (91.76–99.71%)	85.19% (66.27–95.81%)	6.59 (2.67–16.29)	0.03 (0.01–0.11)	95.40% (88.64–98.73%)	92.00% (73.97–99.02%)
>5,000	15.61	96.10% (89.03–99.19%)	71.43% (53.70–85.36%)	3.36 (1.99–5.69)	0.05 (0.02–0.17)	88.10% (79.19–94.14%)	89.29% (71.77–93.67%)
>10,000	30.46	91.55% (82.51–96.84%)	75.61% (59.70–87.64%)	3.75 (2.18–6.46)	0.11 (0.05–0.25)	86.67% (76.84–93.42%)	83.78% (67.99–93.81%)

[a] percentage positive at each threshold: TWB > 1,000: 75.89% (95% CI: 66.90–83.47%). TWB > 5,000: 68.75% (95% CI: 59.30–77.17%). TWB > 10,000: 63.39% (95% CI: 53.76–72.29%).

[b] The range of CT3 serum scores for the three locations were as follows: UK 0.90–80.24, US 1.04–101.62, France 1.86–14.23. The percentage of horses in the TWB categories were as follows: UK - <14.37 (<1,000 TWB) = 20%, 14.37–30.46 (1,000–10,000 TWB) = 12%, >30.46 (>10,000 TWB) = 68%; US - <14.37 (<1,000 TWB) = 17%, 14.37–30.46 (1,000–10,000 TWB) = 13%, >30.46 (>10,000 TWB) = 70%, France - <14.37 (<1,000 TWB) = 100%.

In all foals, CT3-specific IgG(T) was not detected before suckling and, by the third day, foals demonstrated CT3-reactive antibody in sera after consuming colostrum. In most mare/foal pairs, CT3-specific IgG(T) levels in foals after suckling aligned with those measured in sera taken at the same timepoint from the respective dam. After acquisition of maternal IgG(T), in all cases, CT3-specific IgG(T) dropped in foal sera over the next 30–60 days, followed by an increase, indicating foals were ingesting grass infected with cyathostomin larvae. By 120 days, CT3-specific IgG(T) in all foals exceeded the serum score cut-off threshold for 1000 TWB (14.37), remaining above this until the end of the study. Individual mares had differing CT3-specific IgG(T) throughout the study. The group were stabled in December 2013 and, apart from two mares treated with ivermectin, all received moxidectin. The effect of moxidectin treatment on CT3-specific IgG(T) was variable. In five foals, IgG(T) fell rapidly post-treatment. In the other four, antibody remained high. In most foals, CT3-specific IgG(T) increased when they returned to pasture. Of the seven mares given moxidectin, CT3-specific IgG(T) reduced in four. Treatment with ivermectin had a variable effect on CT3-specific IgG(T). Two mares received pyrantel embonate on 2–3 occasions, after which small reductions in CT3-specific IgG(T) were observed.

3.3. Assessment of CT3 serum scores in equine populations based in the UK, US and Germany

CT3 serum scores were assessed with respect to nematode transmission risk in populations where there were significantly different levels of strongyle egg shedding between populations: German Yard FEC versus Charity Herd FEC, $P < 0.0001$; German Yard FEC versus University Herd FEC, $P < 0.0001$; Charity Herd FEC versus University Herd FEC, $P = 0.0043$. Three categories of individual FEC (0 EPG, 1–199 EPG, >200 EPG) were then considered with respect to the proportion of horses above or below the serum score threshold of 14.37 (1,000 TWB), used to assign horses as ELISA-'negative' or -'positive' in each egg shedding category. Also considered, was a sub-group of individuals with serum scores > 50.0 within the ELISA-positive cohort. Serum scores above this value were designated 'off-scale'; these represent a useful indicator of high CT3-specific IgG(T) in individuals. The results are summarised in Table 3.

The proportion of horses classified as serum score-positive in each population varied, with highest numbers positive in the Charity Herd (80%), followed by the German Yard population (60%), with lowest levels (22%) in the University Herd (Fig. 1A). There was no significant difference in serum scores between populations. No horses in the University Herd reported with a FEC of > 200 EPG, with 77% FEC-negative (Fig. 1B); of these, 81% had a negative serum score (Fig. 1C). Of the 23% FEC-positive horses in this population, 71% had a negative serum score, suggesting burdens of < 1,000 cyathostomins in these horses, indicating low nematode transmission. In contrast, the Charity Herd had the lowest proportion (27%) of horses FEC-negative (Fig. 1B); only 41% of these returned a negative serum score (Fig. 1C). Of the 74% of horses in the Charity Herd that were FEC-positive (15% with FEC > 200 EPG), 87% had a positive serum score, indicating high cyathostomin transmission in this population. In the German Yard population, 36% of horses were FEC-negative and, of these, 69% returned a negative serum score. This group had the highest proportion of horses with FEC > 200 EPG and, of these, 86% were above the serum score threshold for 1,000 worms.

When all horses in the three populations were analysed together (Fig. 1D), 68% of horses in the FEC-negative category demonstrated ELISA results lower than the serum score 14.37 cut-off for 1,000 worms. In contrast, 29% were negative at this serum score threshold in the 1–199 EPG FEC category, and, in the

Table 3
Summary of faecal egg count (FEC) results and CT3 serum scores for three equine populations. In all cases, FEC testing was undertaken by the collaborating group; the average FEC and range for each population is shown here in eggs per gram (EPG). The CT3 ELISA was performed at Austin Davis Biologics (UK); the average and range of CT3 serum scores are shown for each population. Serum scores are calculated from relative concentrations of CT3-specific IgG(T) derived from ELISA absorbance and use of ELISA calibration curves. The number and percentage of horses above the cut-off value selected as a positive serum score (14.37, 1,000 TWB threshold) is indicated. A sub-group of horses within the CT3 ELISA-positive group, which measured as off-scale (serum score > 50) is also included for comparative purposes.

Group [a] (equids per group)	Most recent anthelmintic treatment	Mean FEC in EPG (range) [b]	Mean CT3 serum score (range)	Percentage positive (serum score > 14.37)	Percentage off-scale (serum score > 50.0)
German Yards (n = 479)	Various 1 month+	226 (0–2,935)	28.05 (-1.30–77.46)	60%	21%
Charity Herd (n = 147)	Targeted treatment programme; interval dependent on test results	113 (0–1,775)	39.42 (-1.07–74.45)	80%	39%
University Herd (n = 93)	10 months	8 (0–180)	10.47 (-0.51–73.18)	22%	4%

[a] Groups: German yard (n = 47 yards) residents under various helminth management regimes (German Yards), Horse Trust residents based in Buckinghamshire, UK (Charity Herd), university teaching horses from California, USA (University Herd).
[b] FEC methodology used in each population: German Yards group - mini FLOTAC method (Cringoli et al., 2017), Charity Herd – modified McMaster method (MAFF, 1986), University Herd – mini FLOTAC method (Cringoli et al., 2017).

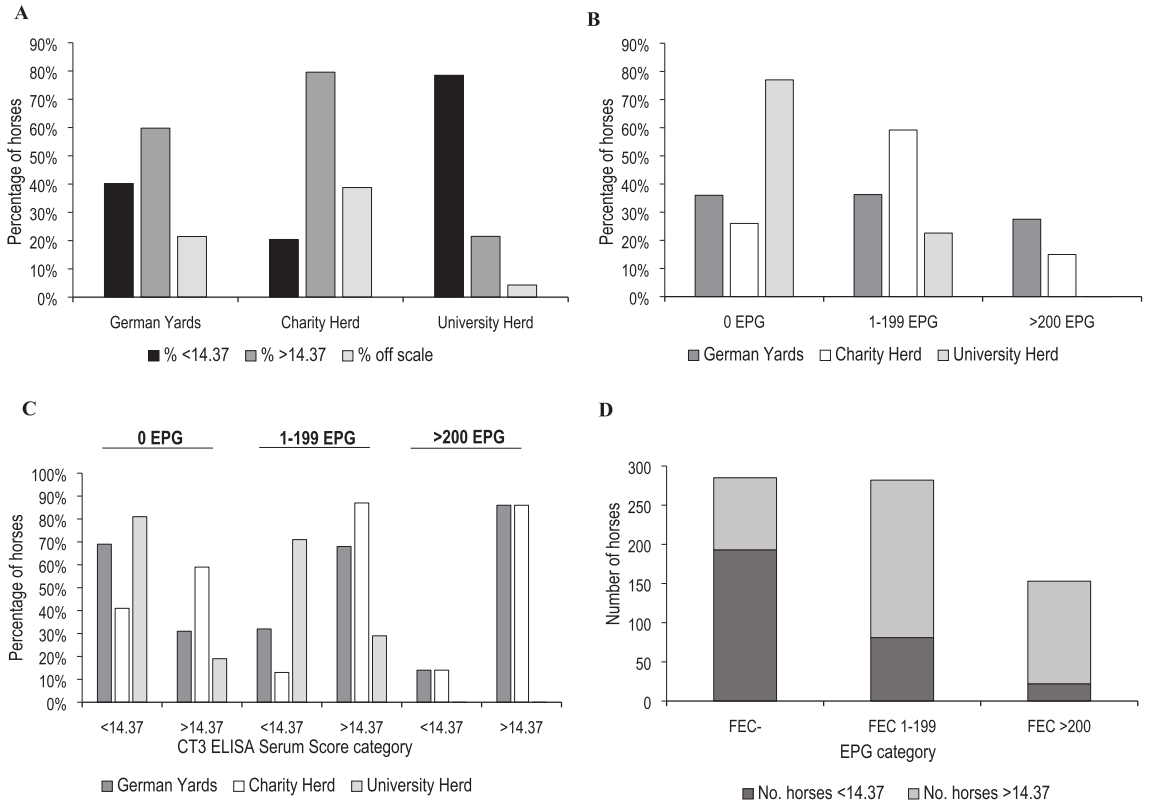

Fig. 1. CT3 serum score and faecal egg count (FEC) distributions in 47 German Yards (n = 479 horses), UK Charity Herd (n = 147 horses) and US University Herd (n = 93 horses). (A) CT3 serum score distributions. Graph depicts the proportion of horses below and above the serum score cut-off of 14.37 (for a 1,000 total worm burden (TWB) threshold). The sub-group of CT3 serum score-positive horses that tested above the off-scale threshold (>50) is shown for comparative purposes. (B) Faecal egg count (FEC) distributions. Graph depicts proportion of horses in each category: 0 eggs per gram (EPG), 1–199 EPG, >200 EPG. (C) CT3 serum score distributions compared between horses grouped according to egg shedding category (0 EPG, 1–199 EPG, >200 EPG). (D) CT3 serum score distributions in the entire cohort, grouped according to egg shedding category (FEC-negative [FEC-], 1–199 EPG, >200 EPG).

FEC > 200 EPG category, only 14% of horses were below this serum score. When all horses were compared, there was a significant difference between FEC-positive and FEC-negative horses in relation to the serum score threshold, with more FEC-negative horses below the 14.37 threshold (193/284: 70%) compared with FEC-positive horses (103/435: 24%) (P < 0.0001). Similarly, significant differences between these two categories (FEC-negative versus FEC-positive) were observed within the Charity Herd (P = 0.0004) and German Yard (P < 0.0001) populations. This was not the case for the University Herd (P = 0.3614), in which a low proportion of individuals were FEC-positive (21/93, 23%). A significant difference was observed when horses were categorised as FEC < 200 EPG versus ≥ 200 EPG groups; significantly more horses with FEC < 200 EPG were below the serum score 14.37 cut-off (274/556: 49.3%) compared with those with FEC ≥ 200 EPG (22/153: 14.4%) (P < 0.0001). The difference at the 200 EPG threshold in the number of horses with < 14.37 serum score was significant in the German Yard group (P < 0.0001), but not in the Charity Herd (P = 0.3778) or University Herd (P > 0.9999).

The German Yard population provided an opportunity to analyse relationships between serum scores and FECs on individual yards and the effect of age (Fig. 2). More horses aged 0–3 years

(58%) had FEC tests results ≥ 200 EPG compared with those aged 4–5 years (38%), and those aged > 5 years (24%). Similarly, 96% of horses aged 0–3 years had serum scores > 14.37, compared with 78% of horses aged 4–5 years and 55% >5 years. The Pearson coefficient between serum score and age was 0.483 and between serum score and FEC was 0.393, whilst age and FEC were not correlated (Pearson coefficient −0.121). Combining both age and FEC in a model correctly predicted a negative serum score in 103/191 cases (53.9%) and correctly predicted a positive serum score in 218/269 cases (81.0%). Odds ratios were 1.044 (95% Confidence Interval (CI) 1.015–1.074) for age and 0.997 (CI 0.996–0.998) for FEC, indicating that for each year increase in age, the odds of a negative serum score increased by a factor of 1.044. The odds ratio for FEC indicated that for each one unit increase in FEC, the odds that the serum score would be negative decreased by a factor of 0.997. Overall, effects of these variables were not large, indicated by a Nagelkerke pseudo R^2 value for the model of 0.175; however, the effects of age and FEC were significant.

3.4. Development of logistic regression models for predicting cyathostomin mucosal and luminal worm burden based on CT3 serum scores

To convert the calculated CT3 serum scores to probability values exceeding selected TWB thresholds, separate logistic regression models were developed (Supplementary Table S4). Model performance is shown in Supplementary Table S5. Model-estimated sensitivity values were lower than the equivalent values determined using the serum score cut-off method; 90.6%, 90.9% and 87.3% for the models at TWB < 1,000, <5,000 and < 10,000, respectively, compared with values of 97.65%, 96.1% and 91.5%, determined using the serum score cut-off method (Table 2). Model-estimated specificity values were similar to those obtained using the serum score cut-off method, with the exception of the value obtained at the < 5,000 TWB threshold, where specificity was greater for the model (74.3%) compared with the value (71.4%) obtained using the serum score cut-off method (Supplementary Table S5).

4. Discussion

Control strategies that target cyathostomins with prophylactic treatments have been promoted for many years (Schumacher and Taintor, 2010), with some experts recommending in publicly available guidelines or reviews that all horses receive an annual treatment (moxidectin or 5-day fenbendazole) in autumn/early winter (Rendle et al., 2019) or at the end of the grazing season (American Association of Equine Practitioners' Internal Parasite Control Guidelines; https://aaep.org/sites/default/files/2021–03/Internal_Parasite_Guidelines.pdf). Due to high levels of benzimidazole resistance (Nielsen, 2022), moxidectin is the drug most often recommended for this purpose (Rendle et al., 2019). Such all-horse treatments are likely to contribute to resistance (Matthews, 2008) and a risk assessment based on management history and recent FEC results should always be undertaken to assess if individuals are likely to need larvicidal therapy. Previous options were to advise no treatment based on an assessment of a low risk of pathogenic burdens of larval cyathostomins, or to apply treatment based on an assessment of higher risk of such burdens being present. A third option is to apply the CT3 ELISA, particularly in horses where it is considered that there is lower risk of cyathostomin infective larval transmission. The results of the test will provide a metric on which to base the decision to treat, or not treat, as the ELISA identifies, with high sensitivity, horses with low burdens (<1,000, <5,000, <10,000 cyathostomins) and will be particularly useful in settings where there is excellent pasture hygiene, or in horses that have limited time at pasture (i.e., sport horses), or where climatic conditions are not conducive to nematode survival or larval translation. In this way, the CT3 ELISA fills a gap in the toolbox to support sustainable control by providing an evidence base for anthelmintic application at times when some veterinarians, or their clients, might otherwise apply blanket treatments. Indeed, the opportunity to apply a serum-based test provides the opportunity for veterinarians to engage with their clients in parasite control, especially in countries such as the UK where owners or non-veterinarian prescribers (for example, suitably qualified persons, veterinary pharmacists) may follow locally relevant guidelines (for example, Rendle et al., 2019; https://www.horsehealthprogramme.co.uk/worming-in-autumn/) and apply a blanket larvicidal treatment in autumn/winter. Employing the test will encourage veterinarians to become more involved in anthelmintic treatment decisions as they can base their advice, if they choose, on results of a test that provides data that discriminates low cyathostomin burden horses from those with moderate/high burdens.

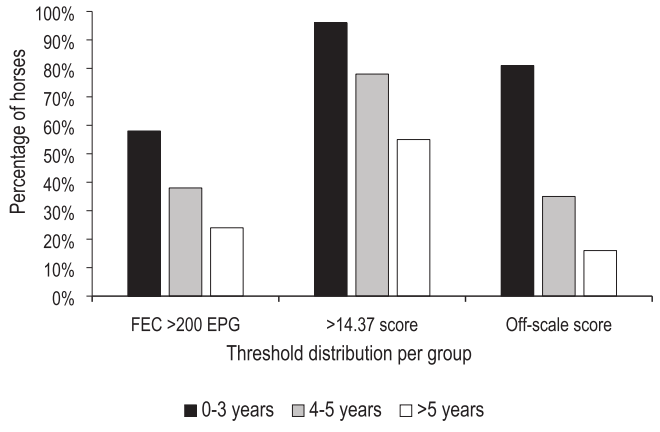

Fig. 2. German Yard cohort categorised by age (0–3 years, 4–5 years, >5 years) for assessing the proportion of horses in each category that are above a faecal egg count > 200 eggs per gram (FEC > 200 EPG), a CT3 serum score of 14.37 (>14.37 serum score) and a CT3 serum score of > 50 (Off-scale score). The sub-group of CT3 serum score-positive horses that tested above the off-scale score (>50) is shown for comparative purposes.

Here, the CT3 ELISA was optimised for commercial application following investigation of all technical steps including antigen sensitisation, dilution of serum and conjugate antibody, selection of substrate and addition of a calibration curve to act as a quality control for every plate. This format was then validated for its ability to diagnose cyathostomin infection at various burden thresholds using gold standard serum samples from 112 horses for which enumeration data were available or were presumed negative. As demonstrated, the test provided excellent diagnostic performance (ROC-AUC values > 0.9), with high sensitivity and specificity for burden thresholds of up to, and including, 10,000 mucosal and luminal cyathostomins, an improvement on the research version, which performed at this level up to a burden threshold of 5,000 cyathostomins (Tzelos et al., 2020). Beyond the 10,000 TWB threshold, ROC-AUC values were < 0.9, possibly associated with the previously observed plateau in cyathostomin-specific serum IgG(T) after repeated infections (Dowdall et al., 2004; Mitchell et al, 2016). Direct comparison of CT3-specific IgG(T) with TWB across the 112 horse validation dataset demonstrated no significant linear correlation, as in previous studies (Tzelos et al., 2020). This may be associated with the fact that serum score values over 50.0 are outside the linear range of the ELISA, as determined by the test calibration curve, and are likely to have confounded correla-

tions between cyathostomin counts and antigen-specific IgG(T) measured across the cohort. ROC-AUC values of < 0.9 were obtained when CT3-specific IgG(T) and mucosal larval counts were compared, as identified previously for the research version of the test (Tzelos et al., 2020). Further development of the CT3 ELISA therefore focussed on threshold cut-off values of up to 10,000 mucosal and luminal cyathostomins, where excellent diagnostic performance was indicated by ROC-AUC analysis. A range of serum score cut-off thresholds were then assessed to develop the ELISA for interpretation relating to cyathostomin burden for use in practice. The selected values, 14.37, 15.61, 30.46 for 1,000, 5,000, and 10,000 worms, respectively, were found to provide sensitivities of 97.7%, 96.1% and 91.5%, and specificities of 85.2%, 71.4% and 75.6%, for the three burden thresholds, respectively.

Time course studies in French foals revealed that, within 3 days of birth, CT3-specific maternal-derived IgG(T) from colostrum was detectable, but varied among foals and in most cases, aligned with CT3-specific IgG(T) measured in dam sera from the same time point. Mare colostrum contains primarily IgG isotype, with less IgA and IgM, all of which are concentrated into mammary secretions (Perkins and Wagner, 2015). The ability to absorb immunoglobulins via specialised enterocytes is greatest in the first 6 h after birth, declining at approximately 24 h, when immunoglobulins can no longer be absorbed (Cervenak and Kacskovics, 2009). Mare-derived antibody provides foals with protection in the first 3 months of life, at which point endogenous antibody production occurs (McKenzie, 2020). In most foals here (6/9), serum scores dropped below the 14.37 1000 TWB cut-off threshold within 3 weeks; however, in foals with high CT3-specific IgG(T) acquired by passive transfer, serum scores did not decrease below this threshold before increasing as a result of their own antibody response to pasture-acquired infection. Based on the results, it was considered that the CT3 ELISA would therefore only provide reliable results (representative of parasite exposure whilst grazing) in foals older than 3 months.

In anthelmintic-treated horses, residual IgG(T) from past infection could have confounding effects on assay accuracy. Since the serum half-life of equine IgG(T) has been reported between 21 (Sheoran et al., 2000) and 35 days (Wilson et al., 2001), this needs to be considered when developing the ELISA for use in practice. Monitoring CT3-specific IgG(T) in the French herd after moxidectin administration provided an insight into temporal dynamics post-treatment. As the herd were stabled for 4 months after treatment, antibody kinetics were not complicated by acquisition of new infections. In 5/9 foals and 4/7 mares, CT3-specific IgG(T) declined rapidly after treatment. In others, IgG(T) did not decline rapidly, or at all. One explanation for these observations is that pre-treatment serum scores were considerably off-scale of the ELISA linear range (serum score > 50), therefore IgG(T) was too high to enable obvious post-treatment reductions to be detected. Alternatively, it could be that moxidectin had not been swallowed/absorbed in sufficient quantity to exert a parasiticidal effect, or, even if administered appropriately, the active ingredient did not kill sufficient cyathostomins to lead to noticeable reductions in specific antibody. In the latter case, this could be associated with the observed variable effect of moxidectin against different mucosal larval stages; in particular, EL3 (for example, Xiao et al., 1994; Monahan et al., 1995, 1996; Eysker et al., 1997; Nielsen et al., 2022). Here, observed differences in serum CT3-specific IgG(T) after treatment could reflect the proportions of mucosal larvae present when moxidectin was administered; animals with more EL3 could have persistent antibody due to a lack of a parasiticidal effect against these stages, which persist or develop to later stages. Horses with a larger proportion of later larval stages and/or adult worms could have more rapid declines in CT3-specific antibody. Based on this data and knowledge of the serum half-life of equine IgG(T) (Proudman and Trees, 1996), to reduce the risk of false positive results, it is recommended the ELISA not be applied until 4 months after treatment, to enable test results to be more representative of a current infection.

In this study, a calibration curve-generated CT3 serum score of 14.37 was used to assess how the test performed in three geographically distinct equine populations. Application of the test at this conservative serum score cut-off for 1,000 cyathostomins would have led to a reduction in anthelmintic application of 41% (296 out of 720 horses), compared with an all-group treatment strategy, which was historically applied in most of these populations. The population with the highest proportion of horses below the 14.37 threshold was the University Herd, in which 78% of animals were observed to have serum scores below this level. These results suggest that the proportion of horses that fall below this serum score cut-off will be determined, at least in part, by levels of cyathostomin transmission which, in turn, is associated with pasture management practices (dung removal frequency, stocking density) and the proportion of horses shedding strongyle eggs at higher EPG. The analysis of the German Yard dataset also indicated that age and FEC contribute to the odds of a negative serum score at the 14.37 cut-off; these factors should be taken into consideration when selecting horses to be tested, and in interpreting the results of the CT3 ELISA.

The results indicate that the CT3 ELISA can be used as a tool by veterinarians to engage horse owners to implement more sustainable control practices. Where recent FEC test results in the group have been low (i.e., 0 - <200 EPG) and excellent pasture management is practiced, veterinarians can choose to advise no anthelmintic treatment, or they can apply the CT3 test to individuals to assess levels of cyathostomin infection, and use the result to inform the treatment decision. In these cases, when selecting the TWB treatment threshold to apply (1,000, 5,000, or 10,000 cyathostomins), clinical evaluation should be undertaken, together with an assessment of the level of infection risk based on knowledge of the yard management practices. On yards where strongyle transmission is judged to be high (i.e., premises with high stocking densities, limited/no pasture hygiene measures, and/or high proportions of young animals), and FEC results are consistently $\geq$ 200 EPG, the test is not recommended for the purpose of informing treatment decisions as many horses are likely to return a positive result above the serum score threshold. The risk assessment in such circumstances indicates that horses are at risk of harbouring pathogenic burdens that may require targeting with anthelmintic.

Although the sensitivity of the univariate logistic regression models was slightly lower than derived by application of CT3 serum score cut-off values, specificity was similar. This type of methodology could be used in reporting of the test results to provide end-users with information on the probability that an individual horse is below a specified burden based on its CT3 serum score and therefore may not be in need of anthelmintic treatment for cyathostomin infection.

Clinical larval cyathostominosis is a serious, albeit relatively uncommon, disease, and published literature regarding pathogenic thresholds of encysted cyathostomin larvae is lacking. At the current time, the CT3 ELISA is not intended as a tool to predict the risk of a horse developing larval cyathostominosis; however, it has been used in outbreaks of acute disease to indicate levels of parasite-specific serum IgG(T) (Walshe et al., 2021). In this report of a welfare establishment, 14/23 horses (1.5–6 years-old) presented with clinical signs of larval cyathostominosis, including signalment in 11 horses in a single week. There was a variety of presentations, the most common being diarrhoea (transient to chronic), weight loss and pyrexia. Of the 14 cases, seven had received an ivermectin product 3 weeks previously. In 12/14 of the clinically affected horses, the signs resolved after anthelmintic

treatment and supportive care; however two horses were euthanased due to the severity of the disease. In both, gross and microscopic examination of the large intestine revealed considerable numbers of cyathostomin L3s and L4s and, on histopathology, a diagnosis of severe necrotising typhlocolitis associated with cyathostomin larvae and bacterial overgrowth was made. Six animals presenting with clinical signs were assessed in the CT3 ELISA; all returned high serum scores, ranging from 53.7 to 70.9 (Walshe et al., 2021). Of note, only one horse in the affected group demonstrated a FEC > 200 EPG, highlighting the need for a test that provides information on the presence of cyathostomins to support clinicians in coming to a differential diagnosis. Ongoing collaborative studies with veterinarians in practice are evaluating use of the CT3 ELISA in larval cyathostominosis cases, taking account of other biomarkers and confounding factors such as plasma protein loss, likely caused by increased intestinal permeability (Love et al., 1992). Because this ELISA has high sensitivity for detecting horses with negligible/low cyathostomin burdens, it also has value as a 'rule out' test to exclude cyathostomins in the aetiology of other intestinal conditions. Feedback from end-user veterinarians indicates that the test is already being used by clinicians for this purpose.

In conclusion, the data presented illustrates the value of a recombinant antigen-based ELISA in identifying the presence of infection and to diagnose burden thresholds above 1000, 5000 and 10000 mucosal and luminal cyathostomins. The test returns a high proportion of negative results in low transmission settings and could have a considerable impact on reducing anthelmintic applications in such populations. The test was launched by Austin Davis Biologics (UK) in 2019, together with user guidelines that were based, in part, on the results here. Since then, the test has been used by veterinarians and researchers across Europe to provide information on cyathostomin burdens in > 16,500 horses to support diagnosis of infection and/or to act as a guide for anthelmintic application.

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This manuscript in dedicated to the memory of Prof. Thomas R. Klei, PhD, former Boyd Professor at Louisiana State University of Veterinary Medicine, USA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2023.07.001.

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