GUIDELINES FOR USE:

SMALL REDWORM BLOOD TEST

CONTENTS

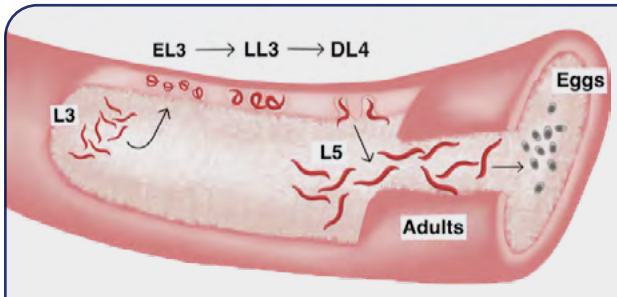
TECHNICAL SUMMARY

Note: *For the purposes of simplifying nomenclature for horse owners, this test has been named 'Small Redworm Blood Test'. For the avoidance of doubt, small redworms are also known as cyathostomins or small strongyles.*

The Small Redworm Blood Test diagnoses cyathostomin infection in horses. The test is an ELISA format which detects IgG(T) antibodies specific to three selected recombinant antigens, representing the most common cyathostomin species as well as all intra-horse stages of the life cycle (**Fig. 1**), including the clinically important encysted larval phase (Dowdall *et al.*, 2002, 2003, 2004; McWilliam *et al.*, 2010; Mitchell *et al.*, 2016; Tzelos *et al.*, 2020; Lightbody *et al.*, 2023).

Validation has been conducted using gold standard samples obtained from horses for which cyathostomin burdens were available. Total worm burdens (TWB) were derived from enumeration of encysted cyathostomin larvae and luminal larvae/adults. High sensitivities and specificities were attained at TWB thresholds of 1,000, 5,000 and 10,000 worms, with area under the curve (AUC) values of Receiver Operator Characteristic (ROC) curves ranging from 0.91-0.96 for the three cyathostomin burden thresholds (Lightbody *et al.*, 2023). ROC-AUC values of >0.9 are universally considered as defining tests with excellent diagnostic performance (Swets, 1988). The test's performance parameters for serum score cut-offs at different TWB thresholds are detailed in the following Open Access peer reviewed publication - *Validation of a serum ELISA test for cyathostomin infection in equines - ScienceDirect.* A summary is shown in Table 1.

Fig. 1. Host stages of the cyathostomin life cycle.



Key: L3 = third stage larvae, EL3 = early third stage larvae, LL3 = late third stage larvae, DL4 = developing fourth stage larvae, L5 = fifth stage larvae.

Table 1. Test performance parameters for the Small Redworm Blood Test.

Parameter[1]	Serum score cut-off for TWB>1,000 cyathostomins: 14.37[2]	Serum score cut-off for TWB>5,000 cyathostomins: 15.61[2]	Serum score cut-off for TWB>10,000 cyathostomins: 30.46[2]
Sensitivity (95% CI)	97.65% (91.76-99.71%)	96.10% (89.03-99.19%)	91.55% (82.51-96.84%)
Specificity (95% CI)	85.19% (66.27-95.81%)	71.43% (53.70-85.36%)	75.61% (59.70-87.64%)

[1] Diagnostic sensitivity is the probability of obtaining a positive test result for a truly positive sample. A highly sensitive test means that there are few false negative results. False negative samples are those that are truly positive but classified as negative by the test at a given TWB threshold. Diagnostic specificity is the probability of obtaining a negative test result for a truly negative sample. False positive samples are those that are truly negative but classified as positive by the test at a given TWB threshold. Confidence interval (CI) is the mean of the estimate value plus and minus the variation in that estimate. The 95% level provides confidence that 95 out of 100 times the estimate will fall between the upper and lower values specified by the CI. For the purpose of diagnostic parameters, 'positive' and 'negative' are defined as results above and below the given serum score threshold, respectively. 'Truly positive' and 'truly negative' are defined as above or below the TWB threshold, respectively, as determined by gold standard diagnosis, enumeration at necropsy.

[2] Serum score cut-off values were selected following Receiver Operator Characteristic (ROC) curve analysis of the ELISA validation dataset and assessing the trade-off of diagnostic sensitivity against specificity over a range of values using GraphPad Prism software (Lightbody *et al.* 2023). The number of cyathostomins (total worm burden, TWB) is the total of larval (mucosal and luminal) and adult (luminal) worms. Selected serum score thresholds for 1,000, 5,000 and 10,000 cyathostomins are shown, with the calculated sensitivity and specificity, for each threshold. 95% confidence intervals (CI) for each performance parameter are included.

USE OF THE TEST

RECOMMENDATIONS

The test can be used by veterinarians to:

- **Diagnose cyathostomin infection in individuals to inform anthelmintic treatment decisions, especially where available advice recommends that all horses be treated annually with a 'larvicidal' anthelmintic**
- **Assess cyathostomin infection prevalence in groups of horses**
- **Support differential diagnosis in gastrointestinal cases, in particular, to 'rule-out' the involvement of cyathostomins**

SKIP TO NEXT SECTION ➤

Rationale for use of the test

For informing treatment decisions, the test identifies, with high sensitivity, horses with burdens of 10,000 cyathostomins and under (Tzelos *et al.*, 2020; Lightbody *et al.*, 2023), so is valuable in circumstances where there is good pasture hygiene, or in horses with limited time at pasture, or where climatic conditions are not conducive to cyathostomin survival/development. Being serum-based, it provides opportunity for veterinarians to engage directly with clients in avoiding blanket treatments, especially where guidelines have recommended that all horses be treated in autumn/winter or at the end of the grazing season with a 'larvicidal' anthelmintic (Rendle *et al.*, 2019; AAEP Guidelines, 2019).

Veterinarians can use this test to assess levels of cyathostomin transmission within herds; by monitoring specific antibody from year to year, the effectiveness of recommended parasite control approaches can be assessed. Monitoring can be considered in autumn/early winter/end of the grazing season as an approach to aid anthelmintic treatment decisions in herds with a low cyathostomin infection risk. In other circumstances, the test can be used at any time of year to assess the effect of a major change in husbandry – for example, after an appropriate period on new clean grazing, or where horse owners/managers are using non-traditional approaches, such as keeping horses on non-grass paddocks or in groups on deep bedding over winter. In these circumstances, the test would provide a useful indicator of cyathostomin transmission.

The test can be used to support veterinarians in making a differential diagnosis in intestinal disease cases. As there is no published literature regarding the level of burden that causes larval cyathostominosis, the test is not intended as a tool to predict the risk of horses developing this disease. The majority of cyathostomin-

infected horses do not show signs of this disease but, in some animals, mucosal larvae accumulate in large numbers (thought to be to the order of 100,000's worms) and emerge to cause larval cyathostominosis. Factors associated with this syndrome include level of parasite exposure, age, season, concurrent disease and treatment history (Giles *et al.*, 1985; Mair, 1993; Reid *et al.*, 1995; Lawson *et al.*, 2023). Diagnosis is challenging and relies on exclusion of other conditions (Reinemeyer and Herd, 1986) and assessment of non-specific blood biochemical and haematological markers (Giles *et al.*, 1985; Mair, 1994). Presentation varies, but cases commonly present with diarrhoea, weight loss and peripheral oedema (Love *et al.*, 1992; Mair, 1994; Mair and Pearson, 1995; Murphy *et al.*, 1997). In Europe, larval cyathostominosis has a well-recognised seasonal occurrence (winter/spring) (Giles *et al.*, 1985) and usually occurs in horses <5 years-old (Reid *et al.*, 1995), but the disease can occur at any age, from 4 months-old (Peregrine *et al.*, 2006) to occurring in aged ponies (Mair, 1993). The disease is most often observed in individuals, but outbreaks can occur (Walshe *et al.*, 2021; Lawson *et al.*, 2023). Larval emergence causes severe damage to the intestinal wall and the disease has a case fatality rate of up to 50% (Love, 1992). The pathogenesis is poorly understood, and the factors involved in triggering mass emergence remain to be established. The disease coincides with the natural period of larval maturation and may only arise when the scale of emergence is sufficiently sizeable to disrupt gut function. Administration of adulticidal anthelmintics has been identified as a predisposing factor (Reid *et al.* 1995). This may be due to treatment killing luminal stages and preventing negative feedback effects on encysted larvae.

There are no metrics available on parasite burdens associated with larval cyathostominosis and there are no studies describing worm counts in fatal cases. This is because enumeration of all worm stages is technically challenging involving transillumination microscopy of tissue and digestion of tissues with larval harvesting (Eysker and Klei, 1999). One study indicated that in a small group of ponies administered infective doses of 3.15-3.9M cyathostomin L3, larval establishment rate in the intestinal wall varied considerably (0.94-39.7%), with only one animal developing larval cyathostominosis (Murphy and Love, 1997). This study serves to highlight the difficulty in predicting disease risk, which is influenced by parasite burden and the individual host inflammatory/immune response. In addition to larval cyathostominosis, these worms have been associated with various types of colic; non-strangulating infarction (Mair and Pearson, 1995), caecocaecal intussusception (Mair *et al.*, 2000), caecal tympany (Murphy *et al.*, 1997) and non-specific mild medical colic (Uhlinger, 1990). There is no information available on the level of larval or adult cyathostomin burden in these cases.

The Small Redworm Blood Test has been employed in acute larval cyathostominosis outbreaks to indicate levels of cyathostomin-specific serum IgG(T) (Walshe *et al.*, 2021). In this report, six animals presenting with acute larval cyathostominosis were assessed in the test. All horses returned high serum scores (53.7-70.9) though only one affected horse showed a strongyle faecal egg count (FEC) >200 EPG, highlighting the role of the blood test in providing supporting information on cyathostomin infection for differential diagnosis in practice. In cases where there is severe hypoproteinaemia, antigen-specific IgG(T) may be low; this should be taken into account when interpreting results of the test alongside other clinico-pathological parameters. It may be worthwhile determining total plasma protein concentration in horses with clinical signs of larval cyathostominosis to rule out false negatives occurring due to low concentrations of IgG(T) present in the sample.

As the test has high sensitivity for detecting horses with negligible/low cyathostomin burdens, it has value as a 'rule out' test to exclude these parasites in the aetiology of other intestinal conditions.

ASSESSING RISK FACTORS TO IDENTIFY SUITABLE HORSES FOR TESTING TO INFORM ANTHELMINTIC TREATMENT

RECOMMENDATIONS

- **To provide information for anthelmintic treatment decisions, horses in low risk environments with recent low FEC results (0-200 strongyle eggs per gram, EPG) should be considered for testing**
- **There is limited value in testing horses to support anthelmintic treatment decisions when they are kept in high risk environments, or if they, or their grazing companions, have recent high FEC results (>200 EPG)**

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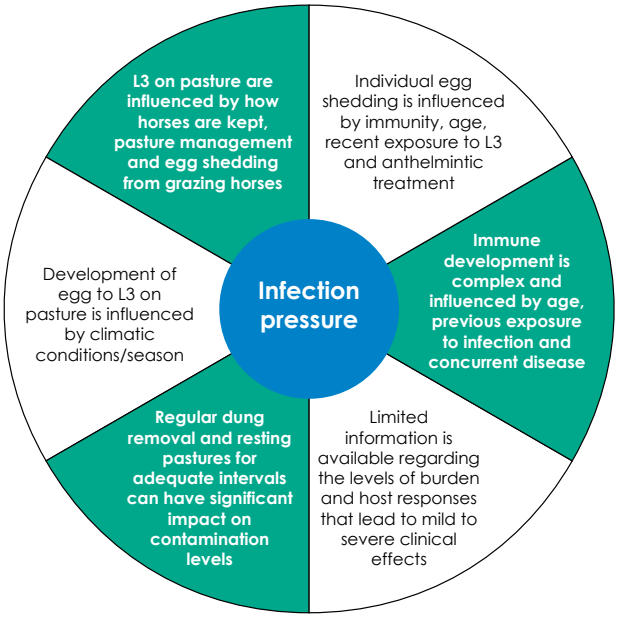
Selection criteria for the use of the Small Redworm Blood test to support anthelmintic treatment decisions

It is essential to assess whether individual horses, or herds of horses, are suitable candidates for testing when using the Small Redworm Blood Test to inform treatment decisions. The rationale behind this approach is based on initial studies (Lightbody *et al.*, 2023) that investigated how the test performed in horses kept under different management/climatic conditions and which exhibited a range of strongyle egg shedding patterns (see below). Full details of these studies can be accessed within the following Open Access peer-reviewed publication - *Validation of a serum ELISA test for cyathostomin infection in equines - ScienceDirect.* Factors to consider are summarised in **Fig. 2.**

Fig. 2. Factors to consider when considering using the Small Redworm Blood Test to inform treatment decisions



A key factor that impacts cyathostomin burden in individuals is infection pressure, i.e., the number of infective third stage larvae (L3) on pasture available for ingestion. L3 levels are associated with the way horses are managed and pasture management such as stocking density and whether or not dung removal has been applied. The number of horses on the paddock excreting strongyle eggs and the level of eggs excreted will have a considerable effect on contamination, especially where effective pasture hygiene is not performed. The number of eggs excreted by individuals is related to the number of female worms in the large intestine, which is influenced by the level of immunity of an individual, the number of worms ingested and treatment history. Note that strongyle FEC correlate poorly with cyathostomin burden, including the levels of adult parasites present. Treatment history will impact egg excretion in terms of the type of anthelmintic used (for example, 'larvicidal' versus 'adulticidal', or if there is a persistent effect of the active ingredient), when the anthelmintic was applied and whether or not it was effective in killing cyathostomins of different stages. Climatic factors (i.e., temperature and rainfall) affect the rate of egg hatching, development of L3 and their ability to translate onto pasture.

These factors must be taken into account when considering using the test for informing anthelmintic treatment decisions, especially:

- FEC test results within the last 6 months (Lightbody *et al.*, 2023)
- Dung removal (i.e., several times a week). This can have a considerable impact on L3 pasture contamination. The application of this practice is a key element in deciding whether or not to use the test to inform anthelmintic treatment.

Testing is not recommended to inform treatment decisions in horses with recent or concurrent FEC results ≥200 EPG. Published studies (Lightbody *et al.*, 2023) that assessed performance of the test in equine groups (3 groups, 719 horses) kept under different conditions and with distinct FEC profiles, indicated that the proportion of individuals above/below the 14.37 serum score cut-off for TWB 1,000 is associated with management and FEC results.

Across all groups tested in this study, application of the 14.37 serum score cut-off would have led to a 68% reduction in anthelmintic treatments in horses with a recent FEC of 0 EPG. There was a significant difference in serum score results between FEC-positive and FEC-negative horses, with more FEC-negative (0 EPG) horses falling below the 14.37 threshold compared with FEC-positive (>0 EPG) horses (P <0.0001). A significant difference was also observed when horses were categorised as FEC <200 EPG versus ≥200 EPG (P <0.0001). These results indicate that the test is best considered for informing treatment decisions when recent FEC in an individual and their grazing companions are low (<200 EPG) and where effective pasture hygiene is practiced.

Where cyathostomin transmission is judged to be high (high stocking density, no pasture hygiene measures, and/or high proportions of young animals) and FEC results are consistently ≥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 thresholds.

In studies where Small Redworm Blood Test results were analysed from groups of horses kept under different conditions (Lightbody *et al*. 2023), 80% of horses in a high transmission setting had serum scores >14.37. A risk assessment in such cases may indicate that horses are at risk of harbouring pathogenic burdens that may require anthelmintic treatment to target all stages of cyathostomins.

Youngsters (1-5 year-olds) are at higher risk of cyathostomin infections and are more likely to be positive in the Small Redworm Blood Test (Lightbody *et al*. 2023).

In horses with limited grazing time, the test is particularly useful in informing whether or not anthelmintic treatment to target all stages of cyathostomins is necessary. For example, analysis of Small Redworm Blood Test results from a mixed purpose sport horse cohort (n=981 horses) showed that the percentage of horses with serum scores below the TWB 1,000 threshold was 62%, 19% of horses were between the 1,000-10,000 TWB threshold, with only 19% of tested horses above the 10,000 TWB threshold

Table 2 provides an example of the risk factors to consider when making the decision to use the Small Redworm Blood Test to inform on anthelmintic treatment.

Table 2. Example risk measurement for assessing individuals for the Small Redworm Blood Test as an aid to informing anthelmintic treatment decisions.

	LOW INFECTION RISK	HIGH INFECTION RISK
Management	• Closed herd, dung removed at least 2-3 times a week, lower stocking density, no young stock (<5 years-old) • Horses with limited grazing time such as racehorses/sport horses	Open herd, no/ineffective quarantine measures, dung not removed/removed less than once a week, higher stocking density, young stock (<5 years-old) present, potential reduced anthelmintic efficacy observed/ anthelmintic efficacy unknown
Faecal egg count results	Concurrent/recent individual or group FEC results <200 EPG	Individual or high proportion of group FEC results ≥200 EPG
Apply test?	YES	NO

Note that although a moderate infection risk category has been made available in other guidelines on parasite control, for the purposes of using this test, infection risk should be assessed as a sliding scale measure from low to high risk.

For low risk horses **(Table 2)**, perform the Small Redworm Blood Test once a year when a blanket cyathostomin larvicidal treatment would previously have been considered. As part of a complete parasite control plan, FEC testing must be carried out throughout March to October to indicate exposure risk/infection and to inform the need to treat during this period to reduce egg shedding in individuals.

RESULTS INTERPRETATION

RECOMMENDATIONS

- **Interpret the blood test result with reference to environmental conditions, management parameters, and recent FEC results**
- **Select the TWB threshold to apply based on assessment of infection risk and pasture management practices**

The test result must be interpreted alongside the clinical and treatment history of the individual or population under assessment. Results are reported as 'serum scores' which are relative concentrations of specific IgG(T) derived from ELISA absorbance and the use of ELISA calibration curves.

The veterinarian is emailed with the serum score for each horse and the reported result should be assessed together with infection risk, based on knowledge of the tested group (age, clinical condition) and the pasture management practices (stocking density, pasture hygiene). The decision on which TWB threshold (1,000, 5,000 or 10,000) to apply can be made alongside infection risk to inform on treatment options.

ADDITIONAL INFORMATION

Antibody half-life considerations

After anthelmintic administration, residual antibody from past infection can have confounding effects on test results. The serum half-life of equine IgG(T) has been reported between 21 (Sheoran *et al.*, 2000) and 35 days (Wilson *et al.*, 2001). To reduce the risk of false positive results, it is recommended that the test is not applied until **4 months after anthelmintic treatment.**

Research has shown that foals' serum IgG(T) responses to cyathostomin infection occur within 6-12 weeks of birth (Murphy and Love, 1997) and, after this time, the maternal antibodies derived from colostrum have diminished. Animals over 3 months are considered appropriate for testing (Lightbody *et al.* 2023).

Horses new to a herd in quarantine

It has previously been recommended that horses should be routinely treated with moxidectin during quarantine, especially if the horse was previously unknown to the veterinarian. A reliable and complete history of the new horse together with current FEC results should be assessed before considering options at quarantine. Submitting a serum sample for Small Redworm Blood Testing could be included as part of this quarantine assessment.

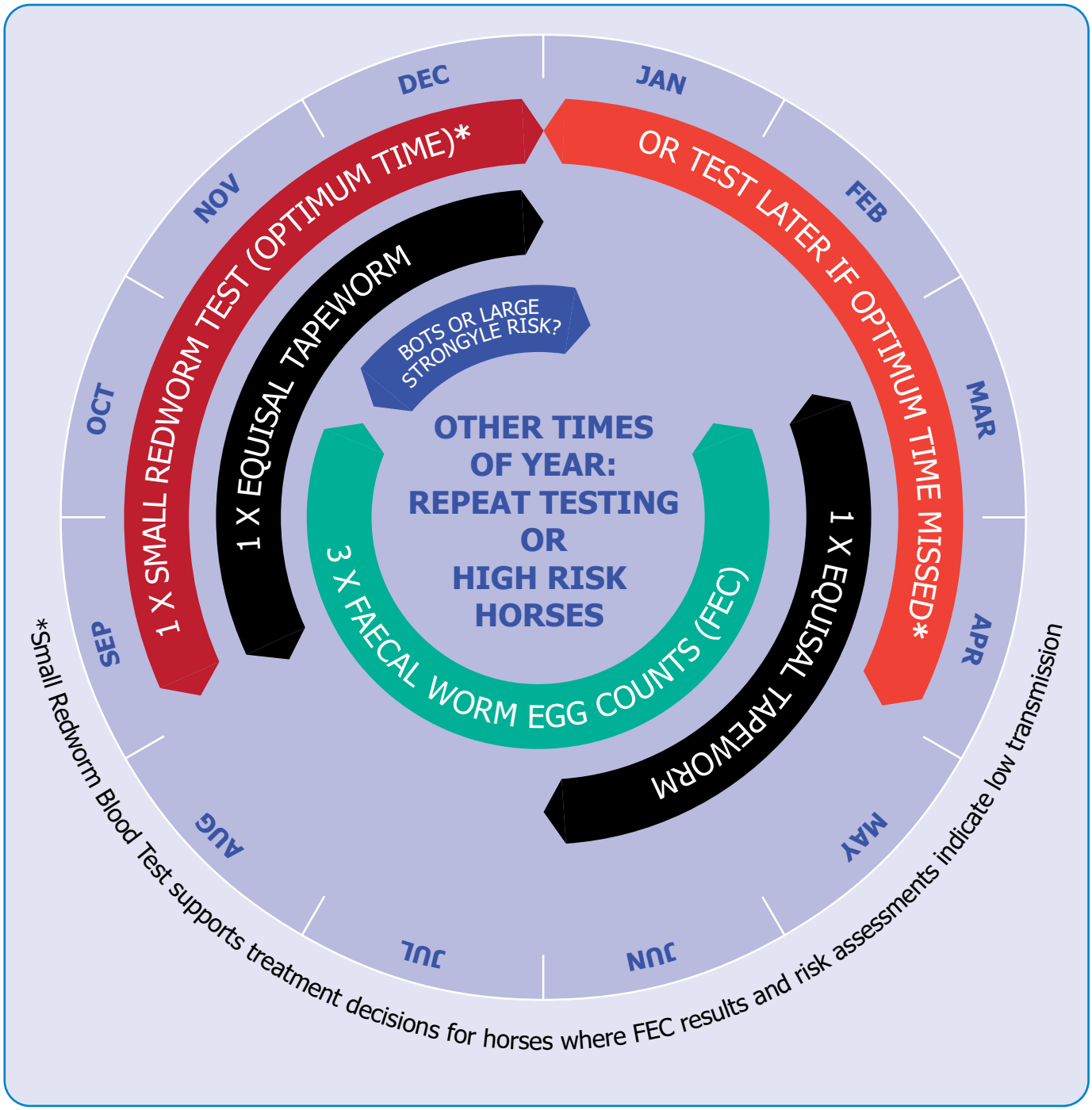
HOW TO USE THE SMALL REDWORM BLOOD TEST SERVICE AT AUSTIN DAVIS BIOLOGICS

1. Register veterinary practice with Austin Davis Biologics by emailing info@austindavis.co.uk
2. Sample submission sheet (pdf) with practice barcode will be issued
3. Determine which horses are suitable for testing
4. Submit at least 0.5 ml serum sample in 1.5 ml microfuge tube together with a sample submission sheet for each sample (a 'whole blood processing fee' will be charged for whole blood submission)
5. Samples will be tested within 2 working days of receiving the sample into the laboratory and results emailed on the day of testing

DIAGNOSTIC-LED WORM CONTROL PROGRAMME

Conducting testing with optimal timing is key to success with diagnostic-led worm control. Fig. 3 illustrates an exemplar seasonal plan for diagnostic-led helminth control in adult horses. All testing protocols must be applied alongside a helminth infection risk assessment for individuals or premises.

Fig. 3. An exemplar seasonal plan for diagnostic-led helminth control in adult horses.



These guidelines are subject to change and will be updated when additional information/ data becomes available.

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The Small Redworm Blood Test was developed in collaboration with Prof. Jacqui Matthews' group at the Moredun Research Institute. This group conducted initial research at University of Liverpool (funded by the Horse Trust), then at Moredun Research Institute (funded by the Horseracing Betting Levy Board, the Thoroughbred Breeders Association and the Horse Trust). The test is covered under patent applications and granted patents originating from patent number PCT/GB 2010/112836.