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Research paper

Semi-quantitative differentiation of cyathostomin larval cultures by reverse line blot

F.N.J. Kooyman^{a,*}, D.C.K. van Doorn^a, T. Geurden^b, J.A. Wagenaar^a^a Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands^b Zoetis—Veterinary Medicine Research and Development, Mercuriusstraat 20, 1930 Zaventem, Belgium

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ABSTRACT

Cyathostomins are the most prevalent horse nematodes worldwide and over 50 species are described. The eggs and the infective larvae (L3) can easily be obtained or cultured from infected horses, but cannot be differentiated morphologically at species level. A reverse line blot (RLB) method based on the hybridization of a PCR fragment with a species specific probe, has previously been developed for the differentiation of individual eggs and/or L3s, but is too labor intensive for large scale studies. In the present study a RLB method on multiple pooled L3s for the semi-quantitative differentiation of cyathostomin larval cultures was developed and validated. First, the probability of the presence of a certain species within a pool was calculated as function of the frequency and the number of L3s within a pool. Ten L3s per pool were found to be optimal. Next, the probability, the chance of occurrence was calculated when 4 pools per culture were used. The probability distributions for 0, 1, 2, 3 or 4 positive pools were transformed into the corresponding median frequency of the cumulative probability: 0.014, 0.04, 0.08, 0.16 and 0.59, respectively. Based on these calculated probabilities, RLB on 10 L3s per pool and 4 pools per sample was validated by estimating the cross-hybridization, precision and accuracy in 3 groups of horses. First, absence of cross-hybridization was confirmed by differentiation of the same L3s (160 L3s from the 4 horses from group 1) in the RLB on individual as well as on pooled L3s. Cross-hybridization was excluded for 9 of the most common cyathostomins. Next, the precision and accuracy were determined by the differentiation of 10 replicates of 3 cultures from 3 horses from group 2 (1200 L3s). The coefficient of variation (CV) was between 0 and 0.90 and the accuracy was between 0.42 and 1.73. A Monte Carlo simulation based on the observed scores and associated probability distributions gave similar results as the use of a fixed median frequency. The LPGs obtained from 276 larval culture counts from a larger cohort (23 horses, group 3) were not significantly different from the LPGs obtained from summation of the LPG per species found by RLB on pooled L3s. The RLB on pooled L3s was found therefore an useful semi-quantitative method for the differentiation of the most common cyathostomin L3, with a workload of approximately one tenth of that of the RLB on individual L3s.

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1. Introduction

Cyathostomins are the most prevalent horse nematodes worldwide and over 50 species are described (Lichtenfels et al., 2008). Adult worms can be differentiated morphologically (Lichtenfels et al., 2008) and the adults of the different species differ in size, predilection site and prevalence (Collobert-Laugier et al., 2002). However, the eggs, first stage larvae (L1), second stage larvae (L2), infective larvae (L3s) and L4s of the cyathostomin species cannot be differentiated by morphological means and therefore many charac-

teristics of these stages of the different species are not well known. Because eggs and/or L3s and to a lesser extent L4s are the most accessible stages, differentiation of those stages can be useful in studies on species-specific anthelmintic resistance.

Ivermectin and moxidectin belong to the macrocyclic lactones (MLs) and nowadays they are the most commonly used drugs for the control of cyathostomins in horses in America and Western-Europe. However, slaughter trials demonstrated reduced efficacy of MLs. In those experiments, efficacy of ivermectin against luminal L4s was only about 10–50% (Lyons and Tolliver, 2013). Efficacy of moxidectin against luminal L4s can be as low as 80%, even when the efficacy against adults in the same horse was 100% (Lyons et al., 2010), but the larvae that survived the treatment were not differentiated. There are also many reports on the shortening of the

* Corresponding author.

E-mail address: f.n.j.kooyman@uu.nl (F.N.J. Kooyman).

egg reappearance period (ERP) after ML treatment in which the eggs and/or cultured L3s were not or only partially differentiated (Geurden et al., 2014; Lyons et al., 2011). Studies have even demonstrated egg reappearance at 14 days after ML treatment (Traversa et al., 2009; Canevar et al., 2013) showing the need for additional studies on the shortening of ERP in order to evaluate the effectiveness of ML treatment. Differentiation of eggs and/or L3s can be especially helpful in these longitudinal studies, where slaughtering of the horses is not an option.

The immature stages can be differentiated to species level by molecular methods (Cwiklinski et al., 2012; Traversa et al., 2007; van Doorn et al., 2010). Those studies used the reverse line blot (RLB), a method based on the hybridization of species-specific probes with an amplified fragment of the intergenic spacer (IGS) region. L3s of 21 of the most common cyathostomin species could be differentiated in this way (Cwiklinski et al., 2012) and van Doorn et al. (2014) used this RLB method to demonstrate that *Cylicocyclus* species are the predominant species in L3 cultures shortly after ML treatment. The differentiation of individual L3s is however labor intensive, and a high through-put methodology would be very useful in larger studies. RLB on pooled samples of cyathostomins were performed and described before (Ionita et al., 2010; Traversa et al., 2010). However, they used pooled fecal samples with a large and variable numbers of eggs per RLB sample or they pooled different samples. In that way they were able to score the absence or presence of a species, but a semi-quantitative estimation was not possible.

The aim of the present study was the development and validation of a semi-quantitative differentiation of cyathostomin L3s by RLB, based on multiple pooled samples with a known number of L3s.

2. Material and methods

2.1. Horses, egg counts and larval cultures

Fecal samples were obtained from 3 groups of horses. Group 1 were 3 horses from a study on the species composition of cultured L3s after ML or pyrantel (PYR) treatment in the Netherlands (details of that study will be published elsewhere) and one free-roaming horse from the nature reserve the Oostvaardersplassen, the Netherlands that was never treated before. Group 2 were 3 horses from the department of Equine Sciences of the faculty of Veterinary Medicine, Utrecht University with an unknown history of anthelmintic treatments. Group 3 were all 23 horses from the same ML-PYR study as group 1. Culturing of L3s was performed as described earlier (van Doorn et al., 2014). The L3s cultured from 25 g faces were stored in 50 ml of tap water at 4 °C. 5 ml of larval suspension was counted, resulting in a detection limit of 0.4 L3 per g faces (LPG).

2.2. Amplification of intergenic spacer (IGS) region by polymerase chain reaction (PCR)

Individual L3s ($n = 40$ per culture) were isolated with 2 μ l culture fluid (tap water) and stored at -80 °C. Individual L3s were lysed with 23 μ l worm lysis buffer/proteinase K (WLB/Prot K, van Doorn et al., 2010) and pooled L3s (10 L3s in 25 μ l tap water) were lysed by addition of 25 μ l 2x concentrated WLB/ProtK. For the evaluation of the cross-hybridization equal volumes of the lysates of the individual lysed L3s were pooled instead of the intact L3s. The mixture was frozen for 15 min at -80 °C, followed by overnight incubation at 56–60 °C. Prot K within the lysate was inactivated the next day at 95 °C for 15 min and the lysates were stored at -20 °C.

For each 25 μ l PCR reaction the optimal amount of 2.5 μ l lysate of the individual L3s and 2.5 μ l of 5x diluted lysate of the pooled L3s was used with 0.2 μ M primer. CY1 (5'GGTCAAGGTGTGTATCCAGTAGAG3') was used as forward primer and CY18 (5'CTTAGACATGCATGGCTTAATC3') was used as 5' biotinylated reverse primer for the amplification of the IGS region (Cwiklinski et al., 2012). PCR reactions were performed in thermal cycler C1000 (Bio-Rad) using Taq polymerase (Thermo scientific) and the following protocol: 10 min at 94 °C and 35 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min followed by a final extension for 7 min at 72 °C. A large batch of lysed L3s was aliquoted and stored at -20 °C and an aliquot of this batch (positive PCR control) and culture fluid (negative PCR control) were included in each PCR run.

2.3. Reverse line blot (RLB)

The RLB is based on the selective hybridization of the IGS amplicon with a membrane bound species-specific probe and is a modification of the method of Traversa et al. (2007). The 20 species-specific probes (for 18 species) and the *cyathostomin* specific probe, all with a 5' C6-aminolinker (800 pmol/lane) were coupled to a membrane (Table 1). For reasons of convenience the genus names were abbreviated to 3 or 4 letters instead of just 1: *Cylicocyclus* (Cyc.), *Cylicostephanus* (Cys.), *Cyathostomum* (Cya.), *Coronocyclus* (Cor.), *Cylicodonthophorus* (Cyd.) *Parapoterios-tomum* (Para.) and *Poterios-tomum* (Pot.). 10 μ l PCR product per lane was used for hybridization. Hybridization was detected with streptavidin-POD (Roche), followed by ECL detection reagent and exposure to Amersham Hyperfilm ECL (both GE healthcare).

2.3.1. RLB on pooled samples

2.3.1.1. Design. The first step in the design of the RLB on pooled samples was the calculation of the probability of a certain species to be present in a pool with a known number of L3s. That probability depends only on the number of L3s per pool and on the frequency of that species. The theoretical probability (p) that a species x is present in a pool of n L3s for a range of frequencies (f) of species x was calculated as $p = 1 - (1 - f)^n$. These and other calculation on the probability were performed in excel (Microsoft office 2010, Microsoft). The results of the calculations are given in Fig. S1. Ten L3s per pool results in the largest difference in probability over the given range of frequencies (probability of 0.075 at the low frequency of 0.78% to a probability of 0.943 at the high frequency of 25%) and therefore, 10 L3s per pool was selected for the RLB on pooled L3s (blue bar in Fig. S1). When more pools were used from the same culture, the calculated probability of the presence of a given species within 1 or more pools also varied with the frequency of that species. Using 4 pools with 10 L3s each, the probability that the given species is present in 0, 1, 2, 3 or 4 pools was calculated. The formulas and outcomes of these calculations are given in Table S1. The probabilities as function of the frequency are shown in Fig. S2. As expected, the probability that the species is present in 0 pools is the highest at a very low frequency and the probability that the species is present in 4 pools is the highest at very high frequencies. The probability that the species is present in 1, 2 or 3 pools is the highest at intermediate frequencies. The frequency that divides the surface below the probability curve into 2 equal parts was determined as the frequency that corresponds with 50% of the cumulative probabilities (Table S1 and Fig. S2, dashed line). The scores (0, 1, 2, 3 or 4 positive pools) for a given species transformed into the median frequency of the corresponding score (0.014, 0.04, 0.08, 0.16 and 0.59, respectively) will result in an observed frequency for that species with an equal chance of being higher or lower than the true frequency. Furthermore, a Monte Carlo simulation was performed on the observed scores

Table 1
Species-specific probes and catch-all probe used in RLB.

Species	Probe—sequence 5'–3'	Reference
<i>Cylicocycclus</i> (Cyc.)		
<i>Cyc. ashworthi</i>	ASH2—GTTCTACTTTATGCAGTGTA	Cwiklinski et al. (2012)
<i>Cyc. insigne</i>	INS2—GTATGTATATGTATCAATGCTTAA	Traversa et al. (2007)
<i>Cyc. nassatus</i>	NAS2—GCAAGAACTTCGCTGAAATG	Traversa et al. (2007)
<i>Cyc. leptostomum</i>	LEP2—ATGTATGCCATTCTTTTATATGTA ^a	Cwiklinski et al. (2012)
<i>Cyc. radiatus</i>	RAD—AGACAGCACTTGCTGTGCCAAT ^b	Cwiklinski et al. (2012)
<i>Cylicostephanus</i> (Cys.)		
<i>Cys. calicatus</i>	CAL2—ACATGCAACACCTGTTCA	van Doorn et al. (2010)
<i>Cys. goldi</i>	GOL—TCTTAGCATCAGGAGAAAT	Hodgkinson et al. (2001)
<i>Cys. longibursatus</i>	LON—GGAGAAATGGTGCGACT	Hodgkinson et al. (2001)
<i>Cys. minutus</i> var. A	MINA1—GGTCACGCTCGATTAACATGCC	Cwiklinski et al. (2012)
<i>Cys. minutus</i> var. B	MINB—GATTTGCAATTAACATACG	Cwiklinski et al. (2012)
<i>Cyathostomum</i> (Cya.)		
<i>Cya. catinatum</i>	CAL1—CTT TTA TCA GCA CTT CTA TG ^c	van Doorn et al. (2010)
<i>Cya. catinatum</i>	CATD5—CGACTAGGCGTACATCATA ^d	van Doorn et al. (2010)
<i>Cya. pateratum</i>	PAT5—CATACAGTTGTAACATTCTCG	van Doorn et al. (2010)
<i>Cya. tetracanthum</i>	TETR—TGGCATCCTTCAAGGTTTCAA	Cwiklinski et al. (2012)
<i>Coronocycclus</i> (Cor.)		
<i>Cor. coronatus</i> var A + B	CORAB—TTCTCAAAGCAAGGGGACTTC	Cwiklinski et al. (2012)
<i>Cor. labiatus</i>	LAB2—GTTCTATTAGGTTGTCTAAGAA	Traversa et al. (2007)
<i>Cor. labratus</i>	LABR2—GCTGAAATGCCGTGTTAGT	Traversa et al. (2007)
<i>Cylicodontophorus</i> (Cyd.)		
<i>Cyd. bicoronatus</i>	BICOR—GCTTCTGATGCGATAAATGACAT	Cwiklinski et al. (2012)
<i>Parapoteristomum</i> (Para.) <i>Para. mettami</i>	MET—GTCTTCTACTCGAGAGGGG	Cwiklinski et al. (2012)
<i>Poteristomum</i> (Pot.) <i>Pot. imparidentatum</i>	IMPAR—GGCTTGATTACGCGCTAGCTAAA	Cwiklinski et al. (2012)
<i>Cyathostomin</i> catch all	Cya-PAN—GAGACTATCCTATGATCGGGT	Traversa et al. (2007)

^a Cross-hybridizes with *Cyc. ashworthi*.

^b Cross-hybridizes with other species, not to be used in RLB on pooled samples.

^c First described by Traversa et al. (2007) as probe for *Cys. calicatus*.

^d Cross-hybridizes with *Cys. longibursatus*, not to be used in RLB on pooled samples.

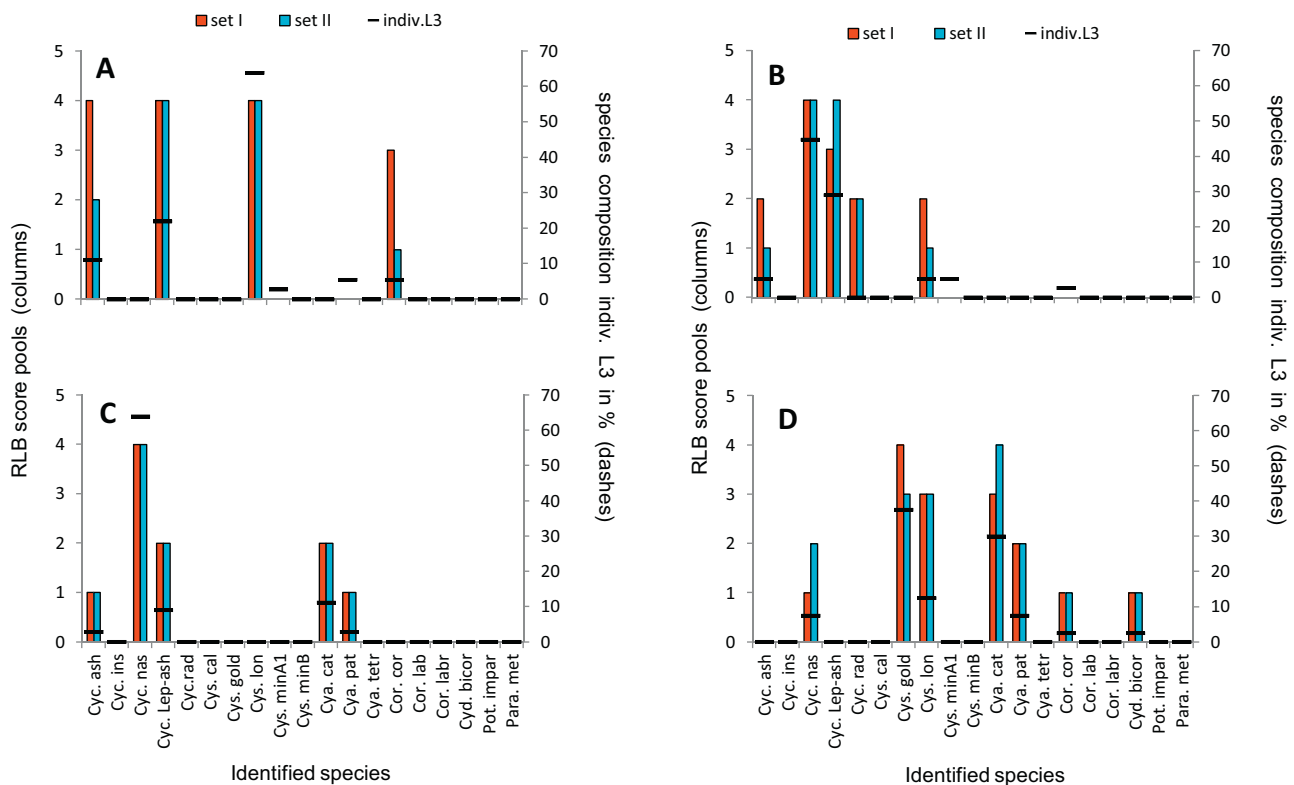


Fig. 1. Estimation of cross-hybridization in RLB on pooled L3s with larval cultures obtained from horses from group 1; horse 2 from farm 1 at day 42 after PYR (A), the same horse at day 42 after MOX (B), horse 8 from farm 2 at day 42 after PYR (C) and the horse from nature reserve Oostvaardersplassen, the Netherlands (D). The RLB score pools (columns, left y-axis) are given and the results of the RLB on individual L3s are given as species composition individual L3 in% (dashes, right y-axis). From each culture, 2 sets (I and II) were prepared and each set consisted of 4 pools with 10 L3s each. Set I and II consisted of the same L3s, but pooled in a different way. For full names of the identified species, see text.

and associated theoretical probability distributions, using the full range of possible values and incorporating uncertainty in the estimates. These theoretical calculations were the basis for the further validation of the RLB on 4 pools with 10 L3s each.

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Probes for RLB on pooled L3s need to be more specific than for RLB on individual L3s, because in the case of individual L3s there is just one L3 present in each RLB lane. Amplicons derived from *Cyc. ashworthi* hybridized not only with the homologous probe (ASH2), but also with the *Cyc. leptostomum* probe (LEP2) (Cwiklinski et al., 2012). Therefore, RLB scores on pooled L3s for the LEP2 probe were counted as *Cyc. ashworthi/leptostomum*. Amplicons derived from *Cys. longibursatus* hybridized not only with the homologous probe (LON), but also with the probe for *Cya. catinatum* (CATD5) (Cwiklinski et al., 2012; van Doorn et al., 2010). Therefore, another probe, CAL1, was used for the identification of *Cya. catinatum* by RLB on pooled samples. This probe was initially used as a probe specific for *Cys. calicatus* (Traversa et al., 2007), but later research identified this probe as specific for *Cya. catinatum* (van Doorn et al., 2010; Cwiklinski et al., 2012). The species known as *Cys. minutus* is possibly a complex of 2 species (Hung et al., 1999) and therefore 2 probes were included in the panel for *Cys. minutus* (Cwiklinski et al., 2012).

A large batch of PCR product obtained from positive controls was divided into aliquots, stored at -20°C and used as positive RLB control in all RLB runs. The spots resulting from the hybridization of the positive PCR control with the *Cys. goldi* specific probe (GOL) were weak, but consistently present in all runs and were therefore used as threshold for all spots from that run. Signals weaker than that GOL spot were considered negative.

2.3.1.2. Evaluation cross-hybridization. Three L3 cultures with a large variety in species composition were obtained from horses from group 1; 3 horses from the ML-PYR study and one horse from the Oostvaardersplassen. From each culture 40 L3s were lysed and the lysates were amplified individually as well as pooled (4×10 L3s). Because the distribution of the different species over the 4 pools can influence the results, the individual lysed L3s were pooled in 2 ways (set I and II). Set I contained the lysates of L3s 1–10, 11–20, 21–30 and 31–40 whereas set II contained the lysates of L3s 6–15, 16–25, 26–35 and 1–5 together with 36–40. All individual and pooled L3 PCR products were assayed with the RLB.

2.3.1.3. Validation of RLB on pooled samples. Three cultures from the 3 horses from group 2 were used for the determination of the precision and accuracy of the RLB on pooled L3s. L3s were assayed in the RLB on individual L3s (96 L3s per culture) and in 10 replicates of the RLB on pooled L3s (10×40 L3s per culture). The frequencies obtained from the RLB on individual L3s were considered the true frequencies. The results from RLB on pooled samples were given in 2 ways: (1) the observed frequency as the mean of the frequencies (0.014, 0.04, 0.08, 0.16 or 0.59) obtained after transformation of the scores (0, 1, 2, 3 or 4 positive pools, respectively). (2) The predicted frequencies as obtained from the Monte Carlo simulation on the observed scores and associated theoretical probability distributions, using the full range of possible values and incorporating uncertainty in the estimates. Precision is expressed as coefficient of variation (CV) of the observed frequencies and the accuracy as the observed frequency divided by the true frequency, so the closer to 1, the higher the accuracy. The precision and accuracy will likely depend on the species and the species composition and because there are 50 cyathostomin species not all variations can be tested. However, all L3s of the common species could be differentiated and this enables the calculation of the total LPGs by the summation of the LPG per species ($\Sigma\text{LPG}_{\text{all species observed}}$). Only a valid differentia-

tion will result in a distribution of $\Sigma\text{LPG}_{\text{all species observed}}$ that is not different from that of LPG obtained by larval counts ($\text{LPG}_{\text{counted}}$). For comparison, the LPGs were also calculated using a simulated ad random frequency per species (0.014, 0.04, 0.08, 0.16 or 0.59) yielding $\Sigma\text{LPG}_{\text{all species simulated}}$. The LPGs were counted and summed from the 276 cultures from the 23 horses from group 3.

2.4. Statistical analysis

The observed frequencies from the replicates of the RLB on pooled samples were transformed into a normal distribution by log transformation and the mean ± 1.96 std. dev represents the 95% confidence interval (CI). The predicted frequency was obtained by Monte Carlo simulation (10,000 replications) using the Microsoft Excel add-in @RISK (Palisade Corp.). Estimates of the predicted mean frequency and 95% credible intervals of each species frequency in the pools were based on the observed scores and associated theoretical probability distributions, thereby modelling the full range of possible values and incorporating uncertainty in the estimates. The parameters for modeling the probability curves in @RISK are given in Table S2. The precision is given as CV (mean divided by the standard deviation) of the observed frequencies. The accuracy is given as the observed frequency obtained from the RLB on pooled L3s divided by the true frequency obtained from RLB on individual L3s. Differences between the true and observed frequencies were tested with the Wilcoxon signed rank test. The linear correlation between the observed and true frequency was calculated after logit transformation. Differences in distribution between the $\text{LPG}_{\text{counts}}$ and $\Sigma\text{LPG}_{\text{all species observed}}$ and also between $\text{LPG}_{\text{counts}}$ and $\Sigma\text{LPG}_{\text{all species simulated}}$ were tested. Differences in the median were tested with the Wilcoxon signed rank test and differences in 95% CI were tested by bootstrapping, with 1000 bootstrap samples. All tests (except Monte Carlo simulation) were performed with IBM SPSS statistics, version 22.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.vetpar.2015.12.009>.

3. Results

3.1. Cross-hybridization

In order to estimate cross-hybridization, the scores from the RLB on pooled L3s were compared with those of the RLB in which the same L3s were used individually (Fig. 1). In the 4 cultures combined, 9 of the most common cyathostomin species were found by both methods (*Cyc. ashworthi*, *Cyc. nassatus*, *Cyc. leptostomum*, *Cys. goldi*, *Cys. longibursatus*, *Cya. catinatum*, *Cya. pateratum*, *Cor. coronatus* and *Cyd. bicoronatus*). Hybridization of the *Cys. radiatus* probe (RAD) was only found with the amplicons derived from the pooled samples (Fig. 1B). Therefore, it was assumed to be cross-hybridization of the RAD probe with one or more heterologous species and the probe was not considered suitable for the RLB on pooled samples. The RLB score for the LEP2 probe (recognizing *Cyc. leptostomum* and *Cyc. ashworthi*) was higher than for the ASH2 probe that only recognized *Cyc. ashworthi*. This suggests the presence of *Cyc. leptostomum* and that was confirmed by the RLB on individual L3s in those cultures. *Cys. minutus* var. A was found in low numbers in the RLB on individual L3s, but none of the pools were found positive. Therefore, *Cys. minutus* A1 numbers are possibly underestimated by RLB on pooled samples.

3.2. Validation

Three cultures from the 3 horses from group 2 were differentiated by RLB on individual L3s (96 L3s) and 10 repeated measurements of the RLB on 4 pools (10×40 L3s), resulting in the

Table 2

Precision and accuracy of the differentiation of 3 larval cultures from 3 horses from group 2 by RLB on pooled L3s. The true frequency (True freq.) was obtained from RLB on 96 individual L3s. The observed frequency (Obs freq) was the mean from 10 replicates of RLB on 40 pooled L3 with a 95% lower confidence (LC) and upper confidence (UC) interval. The predicted frequency (Pred freq) with LC and UC interval was obtained by Monte Carlo simulation (see text). All frequencies are given in percent. Precision is expressed as Coefficient of Variation (CV) of the Obs freq and the accuracy is expressed as Obs freq/True freq.

	Species	True freq	Obs freq (LC–UC)	Pred freq (LC–UC)	CV	Obs freq/true freq.
H I	<i>Cya. catinatum</i>	5.3	3.1 (2.3–4.3)	4.8 (2.9–6.9)	0.58	0.59
	<i>Cya. pateratum</i>	16.8	17.9 (10.6–30.5)	26.0 (16.6–35.0)	0.13	1.06
	<i>Cyc. insigne</i>	27.4	45.5 (30.7–59.0)	49.7 (35.5–63.4)	0.04	1.66
	<i>Cyc. nassatus</i>	17.9	11.2 (7.0–17.9)	16.3 (9.9–22.2)	0.20	0.63
	<i>Cys. goldi</i>	3.2	2.5 (1.7–3.7)	4.2 (2.5–6.3)	0.76	0.80
	<i>Cys. longibursatus</i>	24.2	30.7 (20.8–45.4)	37.4 (25.6–49.1)	0.06	1.27
H II	<i>Cya. catinatum</i>	23.7	20.4 (11.2–37.2)	30.5 (19.8–40.1)	0.13	0.86
	<i>Cyc. insigne</i>	2.1	1.4 (1.4–1.4)	2.5 (1.2–4.3)	0.71	0.67
	<i>Cyc. leptost/Cyc. ash</i>	2.1	1.9 (1.6–2.6)	3.3 (1.8–5.2)	0.87	0.91
	<i>Cyc. nassatus</i>	30.9	14.7 (9.2–23.5)	21.5 (13.4–29.2)	0.16	0.48
	<i>Cys. goldi</i>	3.1	1.9 (1.6–2.6)	3.3 (1.8–5.2)	0.87	0.62
	<i>Cys. longibursatus</i>	14.4	9.1 (6.1–15.6)	14.3 (8.4–19.8)	0.25	0.63
	<i>Cor. Labratum</i>	1.0	1.4 (1.4–1.4)	2.5 (1.2–4.3)	0.00	1.40
	<i>Cor. coronatum</i>	1.0	1.7 (1.4–2.4)	3.0 (1.6–4.9)	0.90	1.73
H III	<i>Cya. catinatum</i>	6.0	2.5 (1.7–3.7)	4.2 (2.4–6.3)	0.76	0.42
	<i>Cys. goldi</i>	3.0	1.9 (1.6–2.6)	3.3 (1.8–5.2)	0.87	0.64
	<i>Cys. longibursatus</i>	92.0	59 (59–59)	57.9 (41.8–73.7)	0.00	0.64

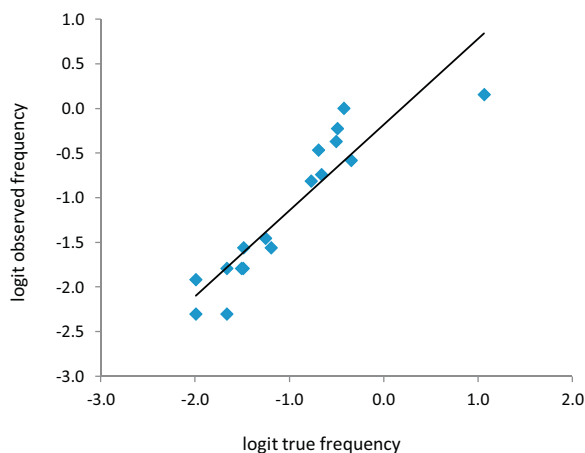


Fig. 2. Linear correlation (black line, $r^2 = 0.836$, $p < 0.001$) between the logit transformed frequency of cyathostomin species estimated by RLB on individual L3s (true frequency, x-axis) with that of the RLB on pooled L3s (observed frequency, y-axis). Same experimental data as in Table 2.

true and observed or predicted frequency, respectively. The precision and the accuracy are given in Table 2. In total 9 species were found and the CV ranged from 0 to 0.9 and the accuracy ranged from 0.42 to 1.73. The observed and predicted frequencies with 95% CI were similar. The true frequency from 11 determinations out of a total of 17, was within the 95% CI of the predicted frequency. Only in one case (*Cys. longibursatus* from horse III) the true frequency was far out of the 95% CI. Three species (*Cya. catinatum*, *Cys. goldi* and *Cys. longibursatus*) were present in all 3 cultures, *Cya. catinatum* and *Cys. longibursatus* had for all 3 cultures different frequencies and different CV and accuracy. *Cys. goldi* had a similar frequency in all 3 cultures and also a similar CV and accuracy. With all determinations combined, no significant differences between the true and observed frequencies or between the true and predicted frequencies were found. Fig. 2 shows that the logit transformed true and observed frequencies were linearly correlated ($r^2 = 0.836$, $p < 0.001$). The logit transformed observed and predicted frequencies were also linearly correlated ($r^2 = 0.9957$, $p < 0.001$, not shown).

Fig. 3 shows that both $\Sigma\text{LPG}_{\text{all species}}$ were linearly correlated with $\text{LPG}_{\text{counted}}$, because both parameters contain the factor LPG. However, only for the $\Sigma\text{LPG}_{\text{all species observed}}$, the relation with the $\text{LPG}_{\text{counted}}$ overlaps the line $y = x$. The $\text{LPG}_{\text{counted}}$ (median 175) was

not significantly different from the $\Sigma\text{LPG}_{\text{all species observed}}$ (median 173), but was significantly different from $\Sigma\text{LPG}_{\text{all species simulated}}$ (median 399, $p < 0.001$). The 95% CI of $\text{LPG}_{\text{counted}}$ (130–226) was also very similar to that of $\Sigma\text{LPG}_{\text{all species observed}}$ (120–217), but different from the interval of $\Sigma\text{LPG}_{\text{all species simulated}}$ (311–513).

4. Discussion

Differentiation of cyathostomin L3s can be performed by RLB on individual L3s (Cwiklinski et al., 2012; van Doorn et al., 2014), however, only a limited number of selected larvae can be differentiated using the individual L3 RLB approach. In the present paper, a RLB with a higher throughput is described. This enables the processing of more samples and hence to generate a more reliable outcome. This RLB uses 4 pools with 10 L3s per pool and was designed and validated for the semi-quantitative estimation of the species composition of cyathostomin cultures.

The design of the RLB on pooled samples was based on the theoretical probability of a certain species being present within a pool of a known number of L3s. We chose to use 10 L3s per pool and 4 pools per culture, as in that case, differences in a wide range of frequencies resulted in large differences in the probability of that particular species being present within a pool. Higher numbers of L3s within a pool will decrease the accuracy in the estimation of the frequency of highly abundant species. For estimation of the frequency of highly abundant species, the use of less L3s per pool can be considered. A species with a frequency of 25% has already a 79% chance to be present in all 4 pools when each pool consists of 10 L3s, indicating that higher frequencies will hardly results into higher scores. On the other hand, the chance that all 4 pools are positive is only 34% when 5 L3s are used. Likewise, for the estimation of the frequency of low abundant species, more L3s can be included in the pools to avoid the absence of rare species. Of course, in order to increase the accuracy more pools can be analyzed but this will increase the workload. In the current set-up with 4 pools of 10 L3s each, the number of PCR reactions and RLB lanes is only 10% of that of a RLB on 40 individual L3s, resulting in an equivalently decreased workload.

The species *Cyc. ashworthi*, *Cyc. nassatus*, *Cyc. leptostomum*, *Cys. goldi*, *Cys. longibursatus*, *Cya. catinatum*, *Cya. pateratum*, *Cor. coronatus* and *Cyd. bicornatus* were found in RLB on pooled as well as in RLB on individual L3s. These are all common species in the Netherlands (Eysker et al., 1992, 1997). No cross-hybridization was

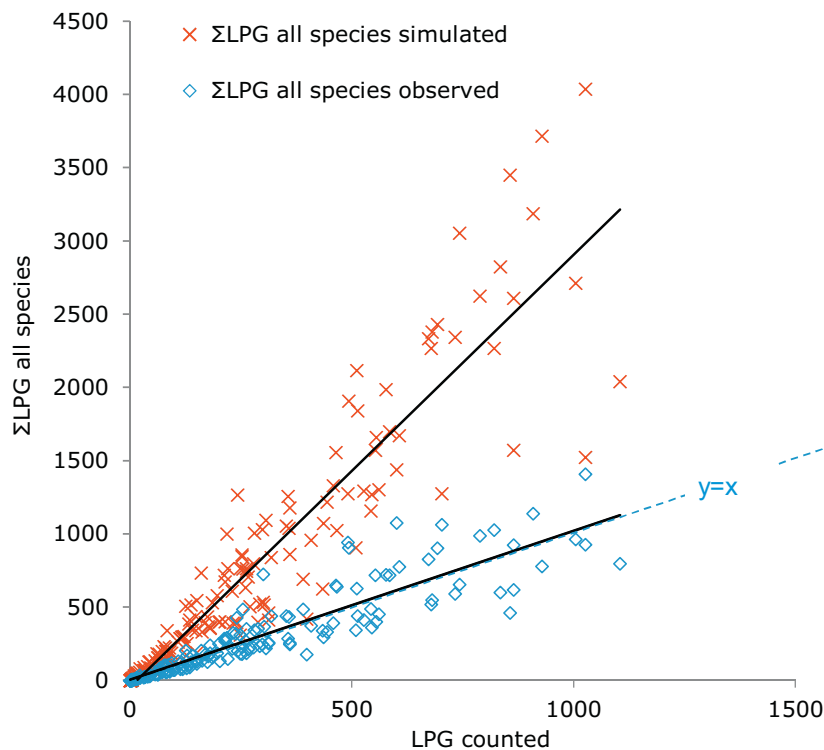


Fig. 3. LPGs obtained from the 276 culture counts (LPG_{counted}, x-axis) from group 3 compared with the LPGs obtained from the summation of the LPGs per species (ΣLPG_{all species}, y-axis) from the same cultures. ΣLPG_{all species} were obtained from simulated random scores (red crosses) or from observed scores from RLB on pooled L3s (blue diamonds). The black lines depict the linear correlation. The blue, dashed line is $y=x$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

found for these species, except for the probe for *Cyc. ashworthi* that also recognized *Cyc. leptostomum*, but this was already known (Cwiklinski et al., 2012). The only cross-hybridization that was found was with the RAD probe that cross-hybridized with amplicons derived from samples without *Cyc. radiatus*. For the RLB on individual L3s that is not a problem, because there is only one species present per lane and the absence or presence of (weak) cross-hybridization is known for all probes (Cwiklinski et al., 2012). For the RLB on pooled samples, however, the presence of many cross-hybridizing L3s within one pool cannot be distinguished from the presence of one or a few individuals of the homologue species *Cyc. radiatus*. Therefore, the RAD probe was not used in the RLB on pooled samples in the present study.

The strength of the RLB signal will differ for the different species because of variation in ribosomal DNA copy numbers and because of variation in efficiency of amplification and hybridization. By using more pools with only a limited number of L3s per pool and only scoring the presence or absence in the pools, the effect of these variations is limited. Above the chosen threshold, the intensity of the spot will not influence the score. This allowed us to compare the frequencies derived from the RLB scores on pooled L3s with that from RLB on individual L3s.

The validation of the RLB on pooled samples was performed by estimation of the precision and accuracy. The CV was between 0 and 0.9 and the accuracy was between 0.42 and 1.73 and the frequencies obtained with the RLB on pooled L3s were positively correlated with the true frequencies. The true frequency of only 7 out of the 17 determinations was within the 95% CI of the observed frequency and in 11 out of the 17 determinations the true frequency was within the 95% CI of the predicted frequency. Many of the determinations in which the true value lay outside the 95% CI were determinations of species with a very low true frequency of only 1 or 2%. A CI of the observed frequency of 1.4–2.4 or 1.4–1.4,

respectively will therefore not include the true frequency, but is nevertheless a good estimation (see for example *Cyc. insignis* in horse II and *Cor. labratum* and *Cor. coronatum* in horse III.). Only in one case (*Cys. longibursatus* from horse III) the true frequency was far away from the CI of the observed and predicted frequency. That sample was exceptional because the true frequency of 92% for a single species is exceptionally high and the current design of the RLB is not well suited for the quantification of species present at such a high frequency. Based on the data obtained from 3 cultures (1488 L3s), the precision and accuracy did not depend on the *cyathostomin* species or species composition and the systematic error was low (mean accuracy = 0.88). Therefore, more samples from more horses within the same group, will likely increase the accuracy of the differentiation. Indications for that were LPG measurements on a larger cohort of horses (23 horses, 276 cultures and 11,040 L3s). The LPG obtained from larval culture counts was not different from the summed LPG of all species as found by RLB on pooled samples and that can only be achieved when the differentiations and estimations of the frequencies are reasonably accurate. That makes the assay very useful for differentiation of larval cultures from large groups of horses, for example before and after treatment, but less so for the accurate analysis of the species composition of the larval culture from one single horse.

5. Conclusion

The RLB on pooled L3s described in the present paper is a reliable semi-quantitative method for the differentiation of cyathostomin L3s cultures and reduces the workload considerably compared to RLB on individual L3s.

Conflict of interests

The study was supported by Zoetis and Thomas Geurden is an employee of Zoetis. The interpretation of the results was not influenced by Zoetis.

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