A Pilot Comparison of Fixatives for Hookworm Real-time PCR

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Abstract:

PCR is increasingly being used in the diagnosis of soil transmitted helminth (STH) infections. Despite this, there are few studies which have evaluated impact of different fecal fixatives on the outcome of fecal helminth qPCR analysis, and none have evaluated the effect of commercial parasitology fixatives commonly used in diagnostic laboratories. We fixed 6 *Ancylostoma* spp. hookworm egg microscopy positive and 3 microscopy negative dog fecal samples in zinc polyvinyl alcohol (Zn-PVA) and Total-Fix. Preservation in 70% ethanol (EtOH) was used as a control. DNA was extracted at timepoints 11, 33, 64 and 94 days and subjected to *Ancylostoma* spp. qPCR. A linear regression model was created to assess the effect of preservative types on the temporal change of qPCR quantification cycle number (Cq) values, accounting for variances among individual animals.

Fixation in 70% EtOH least impacted Cq values over 94 days. Total-Fix preservation yielded a higher Cq overall, but there was no significant difference compared with 70% EtOH fixation. Fixation in Zn-PVA resulted in significantly ($p < 0.001$) higher Cq values than 70% EtOH after only 33 days and loss of amplification at 64 days. Consistent with other helminth fixation studies, 70% EtOH performed very well in preserving hookworm DNA over 94 days. Total-Fix provided a comparable alternative for qPCR analysis for hookworm. Fixation in Zn-PVA resulted in loss of detectable hookworm DNA at 64 days, as determined by qPCR.

Introduction

PCR diagnostics for intestinal helminths are increasingly being employed in both diagnostic and research laboratories around the world. Several papers have studied the efficacy of various PCRs for the diagnosis of soil-transmitted helminth (STH) infection compared to traditional microscopic techniques.[1-4] Others have determined the effects of various disinfectants and fixatives on STH egg biological viability.[5] Few studies have evaluated the pre-analytical effect of commonly used commercial fixatives on the detection of STH by qPCR.

No studies have evaluated two of the most commonly used commercial fixatives, zinc polyvinyl alcohol (Zn-PVA) and Total-Fix (Medical Chemical Corporation, Torrance, CA) for helminth PCR. These fixatives are effective at the preservation of intestinal protozoa morphology[6] and do not share the deleterious effects on PCR of 5% or 10% formalin.[7] One study evaluated the preservation of intestinal protozoan DNA in Total-Fix at room temperature for 24 hours using the commercial Becton Dickinson MAX Enteric Parasite Panel real-time PCR, demonstrating positive results when compared to three other fixatives.[8] Another study successfully detected *Cyclospora cayetanensis* DNA from both Zn-PVA- and Total-Fix-preserved stools by qPCR.[9] Although diagnostic PCRs for helminths are performed on Zn-PVA- and Total-Fix-preserved samples in clinical laboratories and research studies, until now no validation studies have been conducted on the effects of these two fixatives on helminth DNA recovery. This pilot study was conducted to compare fixative impact on DNA preservation at timepoints that reflect duration between clinical sample collection and arrival to the laboratory with remote collection practices in place, as is currently occurring with sample collection for surveillance studies ongoing in the Southern United States. This pilot study determined the effects of two commercial fixatives on the detection of dog hookworm (*Ancylostoma* spp.) DNA by real-time PCR over 94 days.

Methods

Sample collection and preservation

Fecal samples were collected from shelter dogs (*Canis familiaris*) in Jackson, Mississippi. Feces were collected from the concrete floor of the dogs' pen without disturbing the animals and all samples were <15 hours old upon preservation. The present study was deemed exempt by the Animal Use and Care Committee at the University of Mississippi Medical Center, the Institutional Animal Care and Use Committee at CDC, and the Animal Ethics Committee at Federation University.

Fecal samples were screened by microscopy to identify samples positive or negative for hookworm eggs. Preservation was performed on-site by placing fecal samples into 30 mL commercial containers containing either Zn-PVA, or Total-Fix™ (both Medical Chemical Corporation, Torrance, CA). All samples were also preserved in 70% ethanol (EtOH) (Fisher Bioreagents, Waltham, MA) as a reference control for PCR and in 5% formalin for the reference control microscopy egg counts. Feces were preserved at a 1:2 dilution with each fixative. Samples and fixative were homogenized by stirring with a stick and shaking prior to preservation in a room climate-controlled to 20-21°C for up to 94 days.

Fecal microscopy by Mini-FLOTAC

On the day of collection, fecal samples were centrifuged at 500 *g* for 10 minutes, then 2 g of the packed fecal deposit were filtered and homogenized with 38 mL of saturated $NaNO_3$ solution (specific gravity 1.2) in a Fill-FLOTAC device (University of Naples Federico II, Naples, Italy). Two flotation chambers (1 mL each) of a Mini-FLOTAC (University of Naples Federico II, Naples, Italy) device were then filled and the device was allowed to stand for ten minutes to float the eggs. Eggs per gram (epg) of fresh unpreserved feces were then counted under a light microscope at 100x magnification.

106 *DNA extraction and qPCR*

107 DNA was extracted from feces preserved in Total-Fix, Zn-PVA, and EtOH at days 11, 33, 64
108 and 94 (+/- 3 days) after fixation (Supplementary Table S1). Actual days after fixation was
109 used for analysis. One mL of preserved sample was centrifuged, then resuspended in sterile
110 saline and allowed to equilibrate overnight at 4°C. Following this, the sample was centrifuged
111 again, the supernatant removed, and the deposit frozen at -80°C for at least 30 min, followed
112 by flash boiling at 100°C for 10 min. The sample was then centrifuged again and the fecal
113 deposit was added to BeadBug™ tubes (Merck, Darmstadt, Germany) and vortexed on a
114 vortex mixer at 2000 rpm for 2 minutes. DNA extraction was then conducted on a 250 mg
115 aliquot of the centrifuged fecal deposit using a QIAamp® PowerFecal® DNA kit (QIAGEN,
116 Germantown, MD) according to the manufacturer's instructions. DNA was eluted into 50 µL
117 of buffer C6 (QIAGEN, Germantown, MD). The eluted DNA was stored at -80°C and
118 shipped on dry ice to the Centers for Disease Control and Prevention (CDC) for PCR
119 analysis.

120 The *Ancylostoma* spp. PCR for the detection of STH developed by Pilotte, et al.[2] was
121 performed at the CDC. CDC was blinded to the microscopy results. The qPCR assays were
122 performed in a total volume of 25 µL, consisting of 250 nM of each primer, 125 nM of probe,
123 Platinum™ Quantitative PCR SuperMix-UDG w/ROX (Thermo Fisher Scientific, Waltham,
124 MA), and 2 µL of DNA template. Each qPCR assay was accompanied by positive (*A.*
125 *duodenale* genomic DNA) and negative (water and hookworm-free DNA) amplification
126 controls. The qPCR was performed on an AriaMx Real-time PCR System (Agilent, Santa
127 Clara, CA) with the following cycling conditions: 50°C for 2 minutes, 95°C for 2 minutes,
128 then 40 cycles of 95°C for 15 seconds and 59°C for 60 seconds. When analysing the real-time
129 PCR results, the fluorescence threshold was manually adjusted to the same value in all runs to

facilitate congruous comparison of qPCR quantification cycle number (Cq) obtained over time.

Statistical analysis

Statistical analyses were performed in R software version 4.0.3 (R Foundation for Statistical Computing). We fitted a linear regression model to assess the effect of preservative types (70% EtOH, Zn-PVA, and Total-Fix) on the temporal change of Cq values, accounting for variances among individual animals. For this purpose, we included Cq values as the dependent variable, and interaction terms between the days after fixation of the samples and preservative types, and a variable indicating individual animals as independent variables. A p-value of <0.05 was considered significant.

Results

Hookworm egg counts

Six hookworm-positive and three hookworm-negative dog fecal samples were included in the present study. The mean average hookworm egg count of the fecal samples at day 0 was 385 (range 24-588) epg.

Effect of three fixatives on hookworm qPCR results

All *Ancylostoma* spp. microscopy and qPCR results were 100% concordant at the start of the study (i.e., six samples were positive and three were negative by qPCR in all three fixatives). DNA extracts of all hookworm-positive samples showed an increase in Cq on *Ancylostoma* spp. qPCR over time (Figure 1), indicating a decrease in detectable hookworm DNA. This decrease was most notable for samples in Zn-PVA (Figure 1). We found a significant correlation of higher Cq values and the number of days after fixation with Zn-PVA, as compared with interaction with 70% EtOH ($p = 0.032$), suggesting a higher rate of the DNA

155 degradation. This correlation was not observed for Total-Fix. The overall Cq values were
156 larger with samples fixed with Zn-PVA compared with those fixed with EtOH ($p < 0.001$),
157 indicating that Zn-PVA did not preserve hookworm DNA as well as the other two fixatives.
158 At day 11, Zn-PVA-preserved stool samples yielded a mean Cq value 4.4 and 5.9 cycles
159 above that of the samples preserved in 70% EtOH and Total-Fix, respectively (Figure 1). By
160 day 94, these differences had risen to 5.8 and 8.6 cycles above the average values for 70%
161 EtOH and Total-Fix, respectively. By day 64, the DNA of the two Zn-PVA samples with the
162 lowest egg count had no amplification detected at the end point (40 cycles) of the qPCR. For
163 the purposes of statistical comparison between Cq values in different preservatives, these two
164 samples were assigned a Cq value of 40 at days 64 and 94. Individual sample data are
165 presented in Figure 2. Although the Total-Fix had generally higher Cq values than 70%
166 EtOH, the difference was not significant ($p = 0.053$) (Table 1). The R squared value of the
167 model was 0.89.
168
169 *Comparison of egg counts and qPCR results*
170 The *Ancylostoma* spp. qPCR Cq values loosely correlated with the intensity of infection at
171 the lowest intensity infection (dog 1; 24 epg), which consistently yielded the highest Cq
172 values in all fixatives (Figure 2). The Cq values derived from samples with higher egg counts
173 (196-588 epg) were widely distributed in Zn-PVA and Total-Fix and did not visually
174 correlate to the mini-FLOTAC fecal egg counts.
175
176 **Discussion**
177 Previously, studies of fecal fixatives for STH PCR have focused on those fixatives commonly
178 used in research. With increasing use of STH qPCR to complement STH microscopy in
179 surveillance studies, pilot data to validate preservative and its impact on sensitivity of

detection, is essential. The present study represents the first STH PCR analysis of stool preserved in two commercial fixatives commonly used in diagnostic parasitology. EtOH was chosen as a reference control due to its positive performance in previous studies evaluating fixatives for *Ancylostoma* qPCR in research studies.[10,11] While a degree of loss was noted in all fixatives over time, preservation in Zn-PVA at room temperature led to the most rapid loss of detectable hookworm DNA, as measured by qPCR Cq values. This reduction in detectable DNA was significantly more than that seen in 70% EtOH. The effect of preservation in Total-Fix on the temporal change of Cq did not attain statistical significance in our study.

Keiser et al.[10] examined the effects of preservation of feces in 96% ethanol (with and without bead-beating) on the median cycle threshold (Ct) of a multiplex qPCR targeting *Necator americanus*, *A. duodenale*, *Ascaris lumbricoides* and *Strongyloides stercoralis* in Eastern Indonesia. Preservation in 95% ethanol lowered the median Ct for *N. americanus* (n=37) by 1.55, but this was not found to be statistically significant by a Wilcoxon signed-rank test. No *A. duodenale* were present in the samples tested.

Papaiakovou et al.[11] tested various preservatives on human stool samples spiked with 400 *N. americanus* hookworm eggs per gram of feces. The effect on helminth DNA concentration, as measured by qPCR, was determined after preservation in 5% potassium dichromate ($K_2Cr_2O_7$), 95% EtOH, Formalternate, PAXgene, desiccation EtOH-silica gel, FTA cards, and RNAlater, at both 4°C and 32°C, for up to 64 days. Snap freezing at -20°C was used as a control. Results were very similar across preservation methods, with storage at 32°C markedly increasing Cq values over time for all preservation methods except silica beads. Preservation on FTA cards led to a much higher Cq results than other preservation methods. Snap freezing at -20°C was the optimal preservation method.[11]

Ayana et al.[12] used 96% EtOH, RNAlater and 5% potassium dichromate to preserve human clinical samples containing *Ascaris lumbricoides*, *Trichuris trichiura*, and *N.*

americanus. These preserved stools were stored at 4°C for 65, 245, and 425 days, prior to DNA extraction and testing by qPCR. Helminth DNA concentration (as determined by extrapolation from Cq) remained relatively stable in all three preservatives for each species. A slight, but noticeable, decline in helminth DNA concentration was only observed for *T. trichiura*-positive stool samples preserved in 96% EtOH and 5% potassium dichromate.[12]

The data in this pilot suggest that although more degradation is seen across all time points for Total-Fix when compared to 70% ETOH, the degradation seen with Total-Fix may not be significant enough to result in failure to detect infection. In contrast, Zn-PVA showed statistically more degradation across the study compared to both Total-Fix or 70% EtOH, but may be acceptable for storage for ≤ 33 days. At 64 days, a third of Zn-PVA-fixed samples included in the present study did not yield detectable hookworm DNA, while DNA was still detected in all samples fixed in Total-Fix or 70% EtOH.

This pilot study of fixation methods for the preservation of hookworm DNA in feces has several limitations. The study was designed to quickly generate actionable results regarding the suitability of Total-Fix and Zn-PVA for the ongoing surveillance studies in the Southern United States. Thus, it included relatively low number of hookworm-positive samples and it omitted other commercial fixatives, such as EcoFix (Meridian Bioscience, Cincinnati, OH), Proto-Fix (Alpha-Tec Systems, Vancouver, WA) and PrimeStore Molecular Transport Medium (Longhorn Vaccines & Diagnostics, MD).

As hookworm is the primary target of our ongoing surveillance in the South-East of the United States, other STH were not analysed. It should be noted that the results of these studies will not translate into similar findings for *Trichuris* spp., *Ascaris lumbricoides* or *Toxocara* spp., all of which have egg walls resist the diffusion of fecal preservatives far more than hookworm eggs.[13] Similarly, these results cannot be translated for *Strongyloides stercoralis*, which is passed as rhabditiform larvae rather than eggs. It should also be noted

that as 70% ethanol was employed as a reference preservation method in this study, the results are not comparable to prior hookworm DNA preservation studies which employed 95% or 96% ethanol as a reference method.[10-12]

The data set is limited and more data on low egg count specimens is needed. We employed linear regression as our model for evaluating the effect of preservatives on temporal changes of Cq values, although the exact kinetics of DNA decay after fixation was not known. We believe it was reasonable to assume a linear relationship in the present study based on the visual inspection of the graph and high R squared value of 89%, but this remains a potential source of bias. For the purpose of regression analysis, we assigned a Cq value of 40 in dogs 1 and 6 at the 64 and 94 day time points following fixation with Zn-PVA as DNA was not detected within the limit of detection (40 cycle). Samples fixed with Zn-PVA had the highest Cq value in all time points, and Cq value of 40 would be the scenario assuming the least degradation of DNA over time; the statistically significant result with this assumption supports the difference in the rate of degradation, although the degree of degradation cannot be inferred with accuracy. Stool samples from six dogs were included in statistical analyses for temporal changes of Cq values. Although the sample size was relatively small, given the consistency in measurements and plausible trends, we believe that the inference made based on this analysis is reasonable; however, an analysis with a larger sample size may provide results with higher precision. There was some variance in the day of DNA extraction among the preservative types evaluated in the present study (± 3 days). We believe that the variance was minimal, and this limitation was addressed by utilizing the model-based approach assessing temporal changes, although this remains a potential source of bias.

The human hookworm *Ancylostoma duodenale* and the dog hookworm *A. caninum* are genetically very similar, having only a 1.6% distance between the mitochondrial protein coding genes.[14] Thus, it is reasonable to assume that the results found in these dog samples

255 will translate into similar findings for *A. duodenale*. There is a wider phylogenetic distance
256 between *A. caninum* and the other human-infecting hookworms, *N. americanus* and
257 *Ancylostoma ceylanicum*,[14-16] but the very similar biology and morphology of *A. caninum*
258 make it a reliable model for these other human-infecting hookworms.[16] This work should be
259 expanded in the future in a larger cohort of samples with a wider variability in hookworm egg
260 counts, repeated for *N. americanus* and *A. ceylanicum*, and for other common and important
261 medical and veterinary helminths, including *Toxocara*, *Ascaris*, *Trichuris*, *Schistosoma*,
262 *Taenia*, and *Strongyloides*.

263 Despite the limitations listed above, this pilot study presents, for the first time, data
264 supporting the use of Total-Fix for the preservation of feces for hookworm PCR for up to
265 three months of storage. The use of Zn-PVA as a fixative prior to hookworm qPCR may
266 result in loss of detectable DNA concentration between 33 days and 64 days of storage,
267 yielding false negative results. Given the ubiquity of these two fixatives in modern U.S.
268 diagnostic laboratories, such data are important. Results presented herein are limited
269 however, and further studies are needed.

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FIGURES:

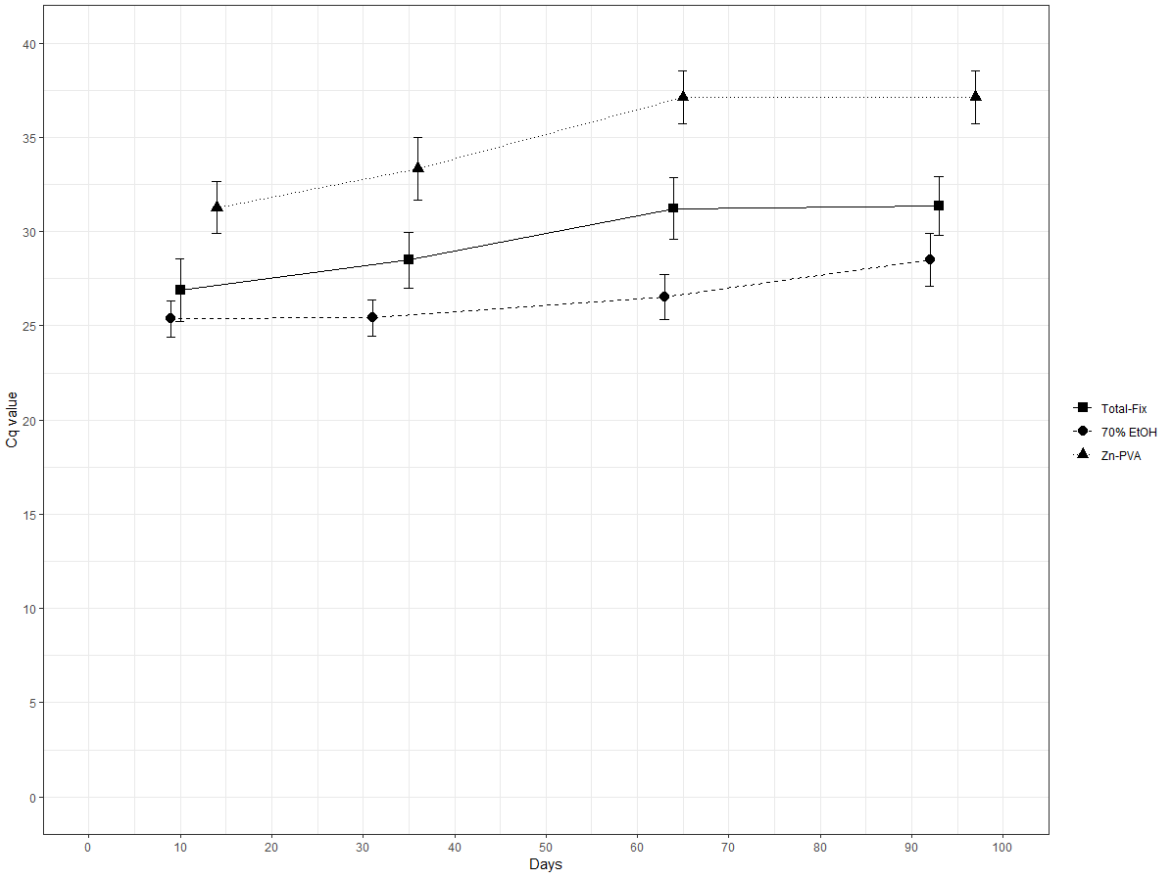

Figure 1: Mean Cq values and standard error bars for six hookworm egg microscopy-positive dog fecal samples preserved in three fixatives when tested using an *Ancylostoma* spp. qPCR over 94 days (days 11, 33, 64 and 94 are +/-3 days).

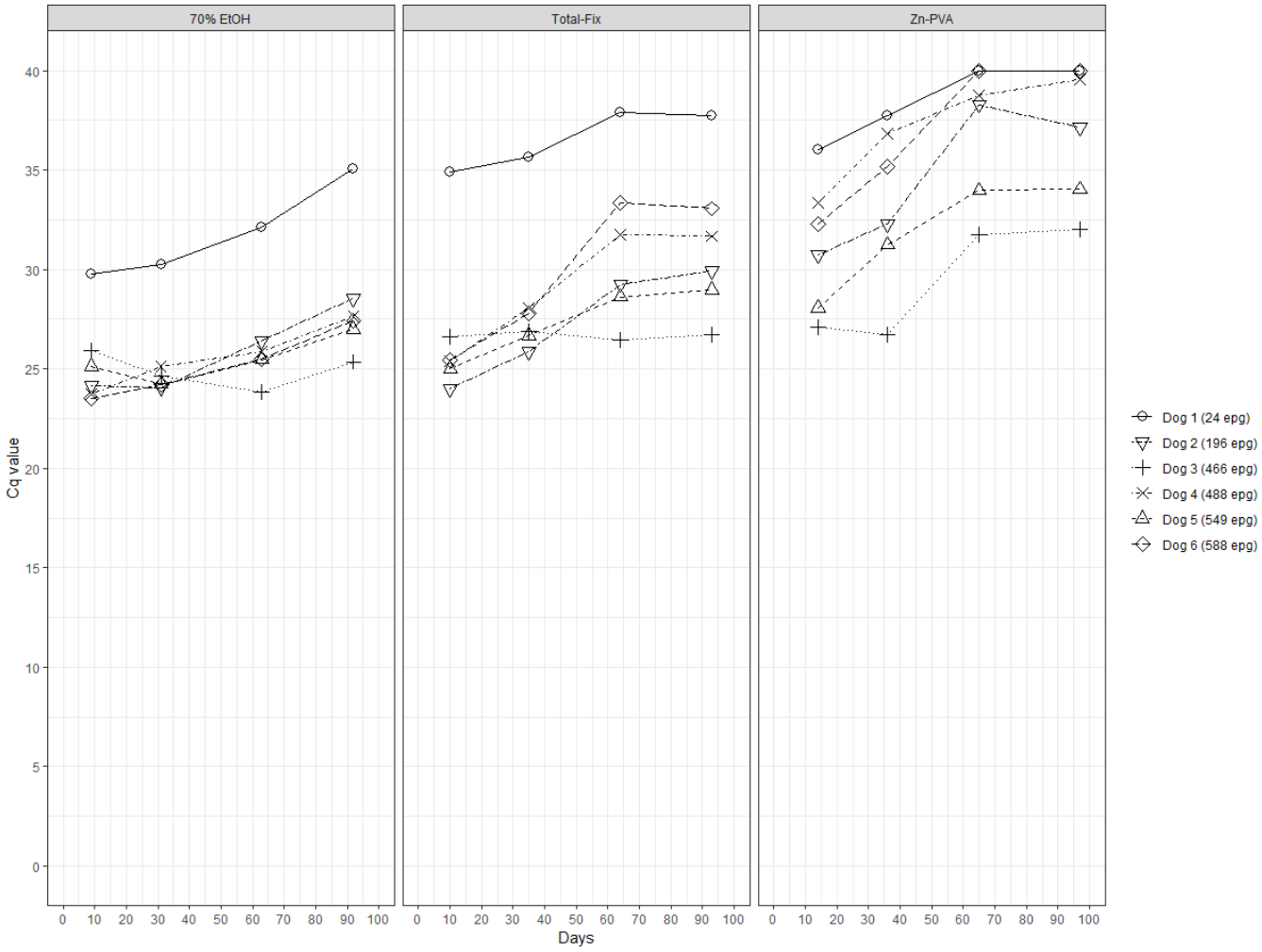

Figure 2: Change in Cq values for six hookworm-positive dog fecal samples preserved in three fixatives over 94 days when tested using an *Ancylostoma* spp. qPCR. The hookworm egg counts on day 0, as determined by saturated salt flotation in a Mini-FLOTAC apparatus, are shown in brackets next to the sample name in the figure legend (days 11, 33, 64 and 94 are +/-3 days). For dog 1 and 6 at 64 and 94 day time points after fixation with Zn-PVA a Cq value of 40 was assigned as DNA was not detected within the 40 cycles limit of detection of the qPCR. epg = eggs per gram of feces as determined in unpreserved samples by mini-FLOTAC at day 0.

Table 1. Results of linear regression analysis of Cq values of six hookworm egg microscopy-positive dog fecal samples preserved in three fixatives when tested using an *Ancylostoma* spp. qPCR (analysis performed in R version 4.0.3).

	Estimates of coefficient (95% Confidence interval) [a]	P value [b]
Days after fixation	0.04 (0.01 – 0.06)	**0.002**
Fixatives		
70% EtOH	Reference	-
Total-Fix	1.97 (-0.03 – 3.97)	0.053
Zn-PVA	6.12 (4.08 – 8.16)	**< 0.001**
Interaction terms		
Days after fixation * Total-Fix	0.02 (-0.01 – 0.05)	0.25
Days after fixation * Zn-PVA	0.04 (0.003 – 0.07)	**0.032**

[a] Model was adjusted for variances among individual animals

[b] Significant values in bold face

Supplementary table S1: Exact number of days following fixation that DNA was extracted from samples in each fixative in this study.

	Days After Initial Fixation Date		
	EtOH	Total-Fix	Zn-PVA
11- Day Extraction	9	10	14
33- Day Extraction	31	35	36
64- Day Extraction	63	64	65
94- Day Extraction	92	93	97