Article

Cost-effective PCR-based identification of *Tunga penetrans* (Siphonaptera) larvae extracted from soil samples containing PCR inhibitor-rich material

Billy L. Amugune [1,†], Abneel K. Matharu [1,2,†], Paul Ouma [1], Francis Mutebi [3], Lynne Elson [4,5], Ulrike Fillinger[1] and Jürgen Krücken [2,*]

[1] International Centre of Insect Physiology and Ecology, Human Health Theme, Nairobi, Kenya; bamugune@icipe.org (B.L.A.); amatharu@icipe.org (A.K.M.); podera@icipe.org (P.O.); ufillinger@icipe.org (U.F.).
[2] Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Berlin, Germany.
[3] School of Veterinary Medicine and Animal Resources, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda; francmutebi10@gmail.com (F.M.).
[4] KEMRI-Wellcome Trust Research Programme, Centre for Geographic Medicine Research (Coast), Kilifi, Kenya.
[5] Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, UK; LElson@kemri-wellcome.org (L.E.).
* Correspondence: Juergen.kruecken@fu-berlin.de
† Co-first author, these authors contributed equally to this work

Simple Summary: *Tunga penetrans* is an invasive flea that causes tungiasis – a neglected skin parasitosis – in both humans and animals. All juvenile *T. penetrans* stages (eggs, larvae, pupa) are found in sandy soil while adults survive on their hosts with females penetrating the skin to breed. In order to understand the ecology of *T. penetrans*, it is important to identify where off-host development of fleas occur by sampling soil for monitoring and surveillance studies. Morphological identification of adult fleas is possible, but due to the absence of a morphological key for the juvenile stages, it is currently impossible to conclusively identify these stages of the flea using morphological features. This study aimed to develop a low-cost PCR-based tool for the identification of *T. penetrans* to be applied in low-income endemic regions. Since juvenile flea stages feed on organic material in the soil, which is known to be rich in PCR inhibitors, this is rather challenging. We tested six protocol combinations based on three DNA preparation methods and two PCR enzymes to determine the most efficient and economical protocol. The developed protocols can be used in future studies and reduce the costs by more than 80% when compared with more conventional approaches.

Abstract: Tungiasis is a neglected tropical disease caused by skin-penetrating female *Tunga penetrans* fleas. Although tungiasis causes severe health problems, its ecology is poorly understood and morphological descriptions of larvae are unavailable. To identify *T. penetrans* immature stages and sites where they develop, diagnostic PCRs are required. However, flea larvae feed on soil organic matter rich in PCR inhibitors. Here, three DNA preparation methods, a soil DNA kit removing inhibitors, a simple ammonium acetate precipitation approach (AmAcet) and a crude lysate of larvae (CL), were combined with amplification by the highly processive FIREPol® Taq or the inhibitor-resistant Phusion® polymerase. Independent of the polymerase used, frequency of successful amplification, C_q values and PCR efficacies for the low-cost CL and AmAcet methods were superior to the commercial kit for amplification of a 278 bp partial internal transcribed spacer-2 (ITS-2) and a 730 bp pan-Siphonaptera cytochrome oxidase I PCR. For the CL method combined with Phusion® polymerase, costs were approximately 20-fold lower than for methods based on the soil DNA kit,

 doi:10.20944/preprints202211.0203.v1

which is a considerable advantage in resource-poor settings. The ITS-2 PCR did not amplify *Ctenocephalides felis* genomic or *Tunga trimammilata* ITS-2 plasmid DNA allowing it to be used to specifically identify *T. penetrans*.

Keywords: Tungiasis; *Tunga penetrans*; molecular entomology; DNA isolation; Phusion® polymerase; FIREpol® Taq polymerase; low-cost PCR,

1. Introduction

Tungiasis is a neglected tropical skin disease affecting humans [1], domestic animals such as pigs, dogs, and cats [2] as well as wildlife [3]. Although the disease is often considered to be only a nuisance and is therefore extremely neglected [4], human disease can be very severe without treatment [5,6]. Domestic animals can also show severe pathology [7–10]. For example, pigs can be infected by several hundred fleas at the same time [9] which can be associated with severe pathology.

Fleas (order Siphonaptera) are blood-feeding parasitic arthropods with holometabolic development [11]. Although adults of all flea species are parasitic, the vast majority of a flea population consists of non-parasitic off-host stages in the environment, i.e. eggs, developing larvae and pupae [12]. Larvae feed on organic matter in the host dwellings or the soil and many species also feed on blood-rich flea faeces or prey on other arthropods including other flea larvae [13].

Host specificity of many flea species is low but there are also many species that are highly adapted to one or a small number of host species [14] and many species at least transiently feed on humans [11]. The most important synanthropic flea species include among others the human fleas *Pulex irritrans* (worldwide), which also infests many animal species, the cat and dog fleas *Ctenocephalides felis* and *Ctenocephalides canis* (worldwide), the rodent fleas *Xenopsylla cheopis* (in the tropics and subtropics) and *Nosopsyllus fasciatus* (in moderate climatic regions), the sticktight flea *Echidnophaga gallinacea* (tropical and subtropical areas) and the sand fleas *Tunga penetrans* and *Tunga trimamillata* [11,15]. Thus, off-host stages of a wide range of flea species can be expected to be present in the environment.

Investigation of flea ecology thus has to focus not only on the availability of hosts but also on suitable sites for off-host development of juvenile stages [16–18]. Among the important environmental parameters, temperature and humidity profiles, available feed for larvae and availability of hosts for the next generation of imagines are the most important. However, currently no estimates about the optima for any of the obvious parameters for off-host development are available for *T. penetrans*. In addition, no systematic studies on spatial or seasonal occurrence of off-host stages of *T. penetrans* has been reported.

For flea control, detailed ecological and physiological knowledge is important in order to develop approaches interfering with the development of off-host stages. Targeting off-host stages has the obvious advantage that drug exposure of humans and domestic animals can be minimised. In order to optimize strategies targeting the off-host stages for tungiasis control, it is important to have detailed information about their localisation to also minimize side effects on non-target arthropods. The fact that many commercialized

 doi:10.20944/preprints202211.0203.v1

drug-combinations targeting fleas of companion animals also target off-host stages emphasizes their importance in the flea developmental cycles and validates them as a suitable target for intervention [19].

In consideration of its importance for human health [4], the knowledge about ecology of off-host stages in Africa is very scarce. Therefore, more studies are needed to generate better information for informed implementation of public health strategies. *Tunga penetrans* was brought by humans to Western Africa (Angola) in the 19th century and it rapidly dispersed to Eastern Africa and to Madagascar with movements of colonial military troops contributing substantially to this geographical range expansion [20]. Although the expansion into and throughout the African continent is largely human driven, the local ecological conditions must support the developmental cycle of the flea. Prevalence of *T. penetrans* in Africa can vary a lot even between sites with close geographical proximity [21]. Assuming that the number of available hosts is not limiting in human settlements, most likely differences in environmental conditions required to support off-host stage development contribute to the observed differences in abundance of *T. penetrans*.

In order to study the off-host ecology of *T. penetrans* and identify development sites for interventions, there is need to identify the species of the flea larvae with certainty. In the absence of valid morphological identification keys for off-host stages, molecular diagnostics are handy in the identification of flea larvae and other off host flea stages collected from various microenvironments since DNA sequences information obtained from adult fleas can be used as reference. Morphological features from specimens identified by molecular techniques can contribute to the generation of morphological keys for *T. penetrans* juvenile stages. However, morphological identification requires experienced personnel and may be error prone. Whilst molecular techniques require robust infrastructure, they are very specific and can thus support identifications based on morphology. To increase the utility of molecular methods in low income countries, the protocols should be as cost-effective as possible.

PCR inhibitors, as found in the soil, may limit the utility of molecular diagnostics. In particular, soil as the habitat from which flea larvae are collected is well known to contain high concentrations of PCR inhibitors [22–24]. There are DNA isolation kits available that are able to remove such inhibitors during DNA isolation and also DNA polymerases that are less susceptible to the effects of inhibitors [25–28]. However, the use of such kits and polymerases further increases the costs for epidemiological surveys. The aim of the present study was to compare different combinations of methods for DNA isolation and PCR on flea larvae collected from human dwellings to achieve robust amplification of target sequences.

2. Materials and Method

2.1 Experimental design

Different combinations of DNA extraction protocols and PCR amplification enzymes were evaluated to identify an efficient, low-cost identification tool for *T. penetrans* larvae collected from soil samples taken from potential flea development sites. The high content of PCR inhibitors provides a challenge for PCR assays targeting *T. penetrans* larvae due to the evident presence of soil and organic matter in their gut (Figure 1). In the present study, three DNA isolation protocols were used: (1) a low-cost DNA preparation protocol using ammonium acetate; (2) a crude flea lysate (CL) protocol (both developed in this study) and (3) a protocol using the NucleoSpin® Soil DNA isolation kit with removal of inhibitors (Macherey-Nagel, Düren, Germany) as standard for comparison. In an initial preliminary comparison, conventional Taq polymerase and the highly inhibitor-resistant

 doi:10.20944/preprints202211.0203.v1

Phusion® DNA polymerase were used. Phusion® DNA polymerase is a thermostable polymerase with high proofreading activity fused to a protein domain binding double-stranded DNA. Due to its poor performance, conventional Taq polymerase was not further evaluated and was replaced by FIREPol® Taq, a modified Taq with unchanged error rate but higher processivity and this was evaluated against Phusion® DNA polymerase. As indicated in Figure 2, DNA samples obtained using the three DNA preparation protocols were then used for amplification using either a hot-start FIREPol® Taq DNA polymerase or Phusion® polymerase. Cost estimates were calculated according to the quantity of reagents needed to process 1000 samples, the estimates were done for both, DNA preparation methods as well as PCR assays. The prices were obtained from recent purchases of the reagents to be used in this study by the International Centre of Insect Physiology and Ecology (*icipe)*, Kenya.

2.2. Flea Sampling

Field sampling for flea larvae was done in Msambweni sub-county, coastal Kenya. Flea larvae were obtained from soil samples that were collected from floors of households with at least one person infected with *T. penetrans*. The Berlese-Tullgren extraction method [29] was used to extract the larvae. It is a method by which soil arthropods are forced by a temperature gradient to move downwards and be trapped by a collection container. This was followed by screening of the arthropod collection under a Zeiss Stemi 508 stereo microscope (magnification 6.3x) to separate other soil arthropods from suspected *T. penetrans* larvae. Reference DNA was obtained from adult *T. penetrans* collected during previous studies [30] and from insectary-reared *C. felis* larvae maintained by artificial feeding at the Institute for Parasitology and Tropical Veterinary Medicine.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

Figure 1. Images of flea larvae taken under a stereo microscope. Presence of soil or organic matter in the flea gut was observed in some larvae (A) but not all (B). The scale bars represent 0.5 mm.

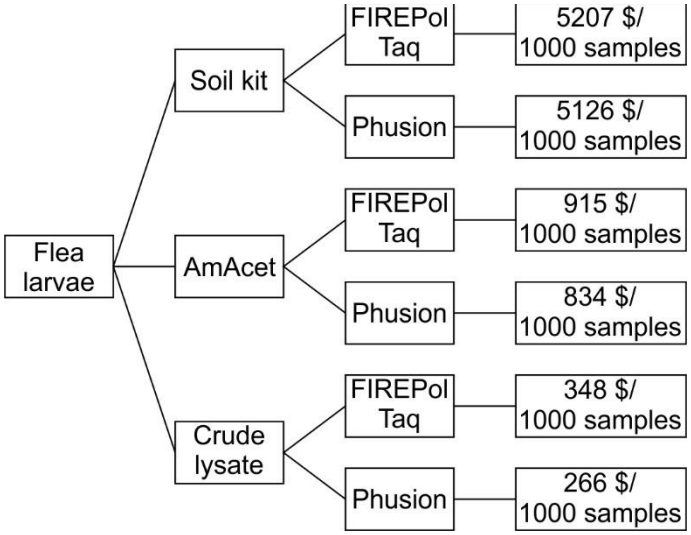

Figure 2. DNA preparation and PCR methods evaluated. Three methods were used to obtain DNA from flea larvae; a soil DNA isolation kit (S-kit), an ammonium acetate precipitation protocol (AmAcet), and a crude flea lysate protocol. Samples from all extraction protocols were used for PCR amplification using either a hot start FIREPol® *Taq* DNA polymerase or the highly inhibitor resistant Phusion® HF DNA polymerase.

2.3. DNA Preparation Methods

2.3.1. NucleoSpin® Soil DNA isolation Protocol

A NucleoSpin® Soil protocol for purification of DNA from soil and sediments was used (Macherey-Nagel, Düren, Germany). Individual larvae were homogenized using beads contained in the kit and a beating device (SpeedMill, Jena Bioscience, Jena, Germany). The isolation followed the manufacturer's protocol and contained an inhibitor removal step optimized for soil samples. The final DNA pellets were eluted with 50 µl elution buffer.

2.3.2. Ammonium acetate DNA Protocol

Larvae were transferred individually to 50 µl tissue lysis buffer (10 mM TrisCl pH 8.0, 0.5% sodium dodecyl sulphate, 5 mM EDTA) in a 1.5 ml microcentrifuge tube and crushed using a bleach-treated pestle. Another 100 µl tissue lysis buffer was then added before 60 µg proteinase K was added. Samples were then incubated at 65 °C for 3 hours. After incubation, 100 µl 7.5 M ammonium acetate was added and the samples mixed by rigorously shaking before placing the samples on ice for 15 minutes followed by centrifugation at 14,500×g at 4 °C for 15 minutes. The supernatant was then transferred to a fresh

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

1.5 ml microcentrifuge tube containing 150 µl of ice-cold 2-propanol, lightly shaken, placed on ice water for 30 minutes and then centrifuged at 14,500×g at 4 °C for 30 minutes. The resulting supernatant was removed and the pellet washed with 150 µl of ice-cold 70% ethanol. Pellets were air-dried overnight. The DNA was finally dissolved in 50 µl of DNase-free water and stored at -20 °C until further use.

2.3.3. Crude Flea Lysate Protocol

Larvae were transferred into individual microcentrifuge tubes containing 50 µl PCR grade water and the tubes were placed in a water bath at 95 °C for 5 minutes. The larvae were then crushed using bleach-treated pestles and incubated at 95 °C for 5 minutes after which they were left to cool down at room temperature (28 °C) before being stored at -20 °C until further use.

2.4. PCR Conditions

2.4.1. PCR Primers

Two different primer pairs were used (Table 1). The *T. penetrans*-specific (TPS) primer pair amplifies an approximately 278 bp fragment of the *T. penetrans* internal transcribed spacer-2 (ITS-2) and is expected to be specific for *T. penetrans* according to manual comparison of ITS-2 sequences of *T. penetrans* and the closely related species *T. trimamillata*. The cytochrome oxidase 2 (cox-2) primer pair has previously been used to amplify a partial mitochondrial DNA fragment from many species of Siphonaptera [31,32] including *T. penetrans* and *T. trimamillata* [33].

Table 1. Primer information. Target gene and primer sequences for both forward and reverse primers.

Target gene	**Primer name**	**Primer sequence** (5'->3')	**Size** ([a]**bp**)	**Annealing temperatures** (°C)	**Reference**
Tunga penetrans ITS-2	TPS-F	TGCTCGACCCGGTGACGGGA	278	FIREPol® Taq 65 Phusion® 69	This study.
	TPS-R	CGCGCAAAGCGTGGAGGTTTCG			
Cox-2	F-Leu	TCTAATATGGCAGATTAGTGC	730	GoTaq 53 FIREPol® Taq 53 Phusion® 53	[32]
	R-Lys	GAGACCAGTACTTGCTTTCAGTCATC			

[a]bp, base pairs

2.4.2. GoTaq DNA Polymerase Conventional PCR Protocol

This PCR was only used in preliminary experiments in the beginning of the study and is not included in the scheme in Figure 2. PCRs were conducted in a final volume of 10 µl 1× Green GoTaq® Flexi Buffer containing 0.5 µM of each cox-2 primer pair (Table 1), 1.25 U GoTaq® Hot Start Polymerase (Promega Corporation, Madison, USA), 2 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (New England BioLabs, Massachusetts, USA).

A BIO-RAD T100™ Thermal Cycler (Marnes-la-Coquette, France) was used for the conventional PCR. After an initial denaturation at 95 °C for 30 s, 35 cycles at 95 °C for 10 s, 53 °C annealing temperature for 30 s, and extension at 72 °C for 45 s were performed before a final extension at 72 °C for 7 minutes.

The resulting PCR products were separated on 2% agarose gels containing Safe View Classic DNA dye (Applied Biological Materials Inc, Richmond, Canada), and the results were documented under UV light using the Syngene InGenius LHR2 Gel Imaging System (Scientific Laboratory Supplies Limited, Nottingham, United Kingdom). A 100 bp Hyperladder (610 ng) (Bioline Reagents Limited, London, United Kingdom) was used as a molecular weight marker.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

T. penetrans genomic DNA from an adult, neosomic flea sample collected during a previous study [30] was included as reference positive control and DNase-free water as no template control (NTC).

2.4.3. FIREPol® Taq DNA Polymerase Real-Time PCR Protocol

PCRs were conducted in a final volume of 10 µl containing 2 µl template DNA, 0.5 µM of each primer pair (Table 1), 1× Hot FIREpol® EvaGreen® (Solis BioDyne, Tartu, Estonia) for real-time PCR. Samples were initially denatured at 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 10 s, annealing at primer pair/method specific annealing temperature (Table 1) for 30 s, and extension at 72 °C for 45 s, before a final extension at 72 °C for 7 minutes was conducted.

Real-time PCR analyses were conducted in a Mic qPCR cycler (Bio Molecular Systems, Upper Coomera, Australia). Fluorescence was measured during the extension period. Melting curves were obtained by heating samples from 72 °C to 95 °C using a 0.1 °C/s ramp. Melting curves were plotted as relative fluorescence units vs. temperature and as the first derivative (-dF/dϑ) of the melting curve.

For all PCR assays, *T. penetrans* genomic DNA from an adult, neosomic flea sample was included as reference positive control and DNase-free water as no template control (NTC). Additionally, for all ITS-2 PCR assays, DNA from cat flea larvae was included as negative control.

2.4.4. Phusion® HF DNA Polymerase Real-Time PCR Protocol

PCRs were conducted in a final volume of 10 µl 1×HF buffer containing 2 µl template DNA, 0.5 M of each primer pair (Table 1), 0.2 U Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Massachusetts, USA), 0.5 mM dNTPs (New England BioLabs, Massachusetts, USA). EvaGreen dye (Jena Bioscience, Jena, Germany) was added to a final concentration of 0.5 µM.

After an initial denaturation at 98 °C for 30 s, 30 cycles at 98 °C for 10 s, annealing primer pair/method specific annealing temperature for 30 s, and extension at 72 °C for 45 s were performed before a final extension at 72 °C for 7 minutes.

Positive and negative controls, and real-time PCR analysis were performed as detailed above (2.5.3.).

2.5. Evaluation of the Specificity of the Tunga penetrans Partial ITS-2 PCR

The *T. penetrans* partial ITS-2 PCR was designed to discriminate *T. penetrans* from other flea species. In order to evaluate specificity, the PCR assays were applied to different template DNAs. Since no specimens of the most closely related flea species *T. trimamillata* were available in the present study, the sequence of the published ITS-2 was artificially synthesised and cloned in the p-SK-A plasmid vector (StrataClone PCR Cloning Kit, Agilent Technologies, Waldbronn, Germany). Plasmids were isolated from *Escherichia coli* cultures using a GenUP™ Plasmid Kit protocol (biotech rabbit, Berlin, Germany) for isolating high-copy-number plasmid DNA from 0.5-5 ml bacterial culture.

The Phusion® HF DNA polymerase real-time PCR protocol was used and DNA isolated from cat flea larvae was also included in the analyses as negative control for specificity.

2.6. PCR Efficacy Analyses for Real-Time PCRs

PCR efficacies were calculated for each sample based on changes of normalized fluorescence vs. cycle number fitting an exponential equation to the data using the LinRegPCR algorithm as implemented in MIC PCR software version 2.8.10 (Bio Molecular Systems, Upper Coomera, Australia).

2.7. Statistical Analyses

Quantification cycles (C_q) and PCR efficacies were compared between different DNA preparation methods after applying the sample PCR protocol using One-Way ANOVA in

 doi:10.20944/preprints202211.0203.v1

GraphPad 5.02. For comparison between different PCR protocols applied to the same set of DNA templates, paired t-tests were conducted. Success rates for PCRs between different protocols were compared using the mid-p exact test as implemented in the tab2by2.test function in the R package epitools 0.5-10.1 using R version 4.1.1.

3. Results

3.1. Initial Comparison of Taq and Phusion® -based PCR Protocols Using Conventional PCR

The soil kit method and the ammonium acetate method were initially compared using conventional PCRs based on amplification of a 730 bp fragment of the cox-2 gene. While amplification was successful for ten out of ten samples for both DNA preparation methods if Phusion® polymerase was used, only six samples were amplified using GoTaq polymerase for the soil kit method and four samples for ammonium acetate method. Given that the successful amplification using GoTaq is approximately 50% compared to 100% using Phusion® polymerase, we decided to use the improved FIREPol® Taq for better comparison with Phusion®.

3.2. Comparison of Different Combinations of DNA Preparation and Real-time PCR methods

The six different combinations of three DNA preparation methods and two different amplification protocols/polymerases were systematically evaluated using real-time PCRs targeting a partial fragment of the ITS-2 region, designed to be *T. penetrans* specific, and a partial cytochrome oxidase 2 fragment. Regarding the costs, there were strong differences between the protocols with by far the highest costs caused by the use of the S-kit for DNA isolation followed by the AmAcet method and only minimal costs for DNA preparation for the CL protocol. For the polymerases, the FIREPol® Taq was slightly more expensive than the Phusion® polymerase. Thus, the combination of S-kit with FIREPol® was by far the most expensive protocol (5207 $/1000 samples) while the combination of CL with Phusion® polymerase (260 $/1000 samples) was almost 20 times cheaper.

3.2.1. Comparison of Combinations of DNA Preparation Methods and PCR enzymes based on a Partial Internal Transcribed Spacer 2 PCR

Representative amplification plots for the TPS ITS-2 PCR using different DNA preparation methods are shown for FIREPol® Taq (Figure 3A) and Phusion® polymerases (Figure 3B). For both polymerases, successful amplification was achieved in more than 80% of the samples (Table 2).

There were no significant differences in C_q values for the same template when the FIREPol® Taq and the Phusion® PCR protocols were compared (Figure 4A). However, C_q values were higher for the S-kit DNA isolation method when the Phusion® polymerase was used. These differences were significant for both comparisons to CL and AmAcet preparation protocols. For the FIREPol® Taq polymerase, the S-kit DNA isolation method also had the highest median and mean C_q values and significant differences were observed when compared with the CL and AmAcet protocols (Figure 4A).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

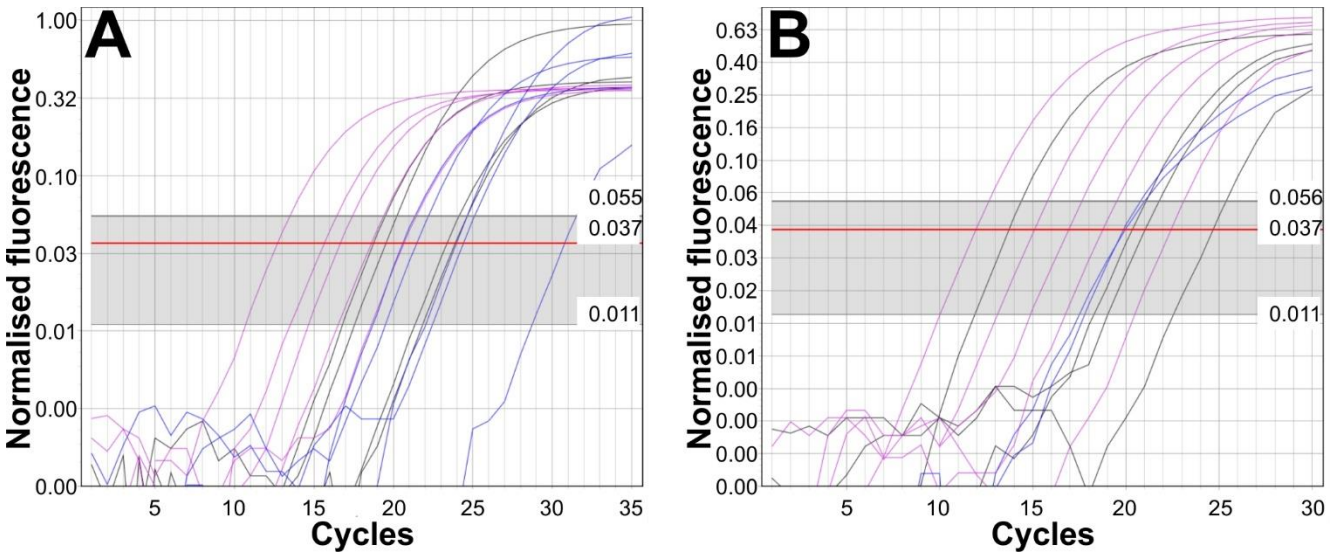

Figure 3. Representative amplification plots for the *Tunga penetrans* using FIREPol® Taq (A) and Phusion® (B) polymerases. The different DNA preparation methods are colour coded: pink for AmAcet, blue for S-kit, and black for CL.

Table 2. Success rate for different PCRs and DNA preparation methods based on 30 replicates.

	FIREPol® Taq				Phusion®			P value		
PCR	**DNA preparation**	**n**	**% pos.**	**95% CI**	**n**	**% pos.**	**95% CI**	**FIREPol® vs. Phusion®[a]**	**FIREPol[b]**	**Phusion®[b]**
Tunga penetrans partial ITS-2										
	S-kit	28	93.3	78.7-98.1	24	80.0	62.7-90.5	0.153	1	0.057
	AmAcet.	28	93.3	78.7-98.1	29	96.7	83.2-99.4	0.619	0.433	0.753
	CL	26	86.7	70.3-94.7	25	83.3	66.4-92.7	0.736	0.433	0.109
Cox-2										
	S-kit	17	56.7	39.1-72.6	17	56.7	39.1-72.6	1	<0.001	0.191
	AmAcet.	30	100	88.7-100	22	73.3	55.6-85.8	0.002	0.112	0.112
	CL	23	76.7	59.1-88.2	23	76.7	59.1-88.2	1	0.005	0.776

n, number of successful PCRs; N, number of PCRs conducted; 95% CI, 95% confidence interval.

[a]Comparison of results for FIREPol® Taq and Phusion® polymerases conducted on the same set of samples.

[b]Comparison between different DNA preparation protocols using the same polymerase using mid-p-exact tests. P values are given from top to bottom for the comparisons of S-kit vs. AmAcet, S-kit vs. CL and AmAcet vs. CL.

In Figure 4B, PCR efficacies as calculated by the LinRegPCR algorithm from the individual amplification plots are shown. PCR efficacies were very similar between the different methods. No significant effect of the DNA polymerase was observed. Comparison of the DNA preparation protocol revealed significantly lower efficacies for the S-kit if used in combination with the Phusion® amplification protocol. However, this was largely ex-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

plainable by three replicates with very low efficacies (below 0.5) in the data set. For FIREPol® Taq polymerase, no significant differences between DNA preparation methods were detected.

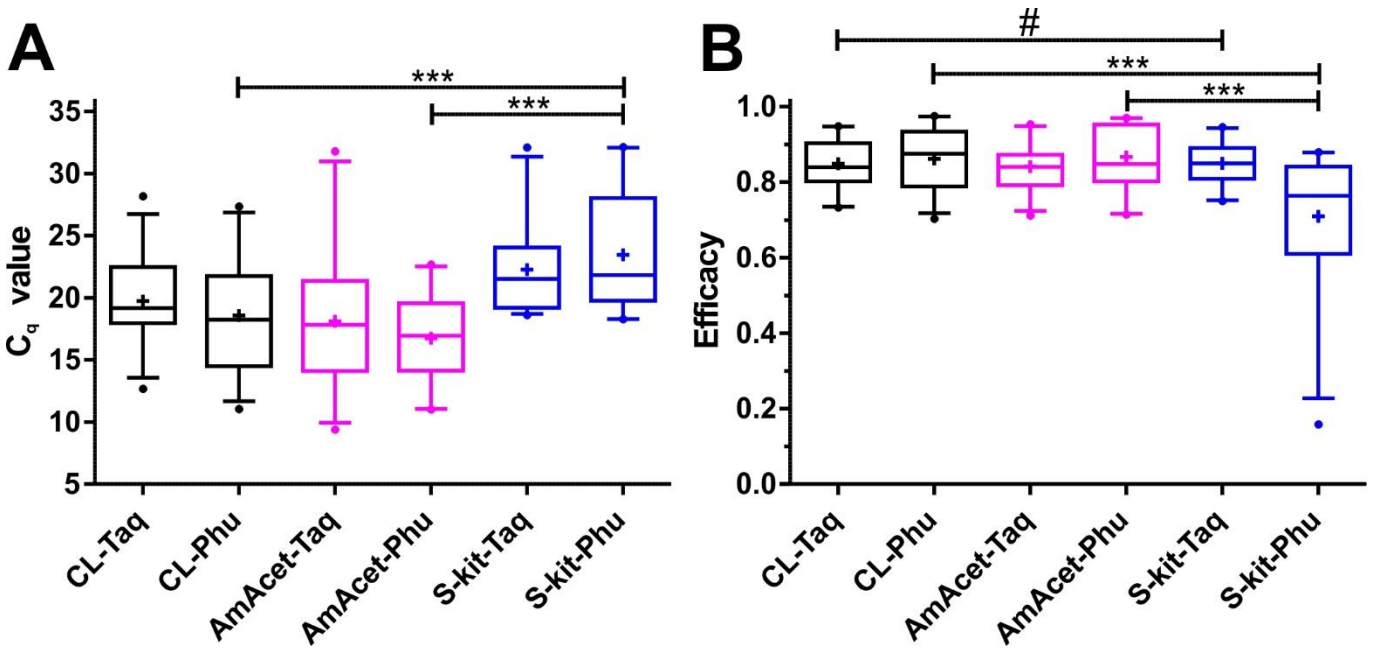

Figure 4. Comparison of cycle of quantification (C_q value) (A) and PCR efficacies (B) between different DNA preparation methods and amplification protocols for a *Tunga penetrans* partial ITS-2 PCR. Template DNA was obtained as a crude lysate (CL) by simple boiling of mechanically cracked larvae, by proteinase K digestion followed by precipitation with ammonium acetate (AmAcet) or using a soil DNA isolation kit (S-kit). Amplification was performed as real-time PCR using either the FIREPol® Taq (Taq) or the Phusion® (Phu) DNA polymerase protocols. Boxplots show medians with interquartile ranges and whiskers represent 5 and 95% quantiles. Outliers are indicated by dots. The mean of all values is shown as a cross. Paired t-tests for the same template DNA using either Taq or Phusion® polymerase protocols did not reveal any significant differences. Comparison between different DNA preparation methods using the same amplification protocol were conducted using One-way ANOVAs. Hashtags were used to indicate differences between DNA preparation methods for the Taq polymerase protocol, while asteriks indicate differences between preparation methods for the Phusion® protocol. #, $p < 0.05$; ***, $p < 0.001$.

3.2.2. Comparison of combinations of DNA preparation methods and PCR enzymes using cox-2 specific PCR

Using the same approach (and the same set of template DNAs) as for the partial ITS-2 PCR, the cox-2 PCR was used to evaluate DNA preparation and amplification protocols. This was done assuming that a PCR with a larger amplification product (278 bp vs. 730 bp) will show more pronounced differences between different protocols.

Table 2 shows a few significant differences in success rate that were not observed for the partial ITS-2 PCR. For the AmAcet DNA preparation method, the FIREPol® Taq protocol was significantly more frequently successful than the Phusion® protocol. Moreover, when FIREPol® Taq protocol was used, AmAcet showed a higher frequency of PCR reactions with a positive amplification than the S-kit and the CL approach. Such differences were not observed for the Phusion® polymerase (Table 2).

Regarding the C_q values, Phusion® polymerase produced significantly lower C_q values than FIREPol® Taq for all three DNA preparation protocols (Figure 5A). Comparison of DNA preparation methods based on FIREPol® Taq polymerase showed lower C_q val-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

ues for the CL and AmAcet methods when compared with the S-kit. For Phusion® polymerase, all comparisons between methods were significant with the lowest C_q values for AmAcet followed by the CL and the S-kit protocol (Figure 5A). Looking at PCR efficacy data, only the combination of the S-kit with the Phusion® amplification protocol revealed significant differences to other protocol combinations (Figure 5B). The S-kit/Phusion® combination showed significantly lower efficacies than the S-kit/FIREPol® Taq protocol and also than the CL and AmAcet. methods in combination with Phusion® polymerase (Figure 4B).

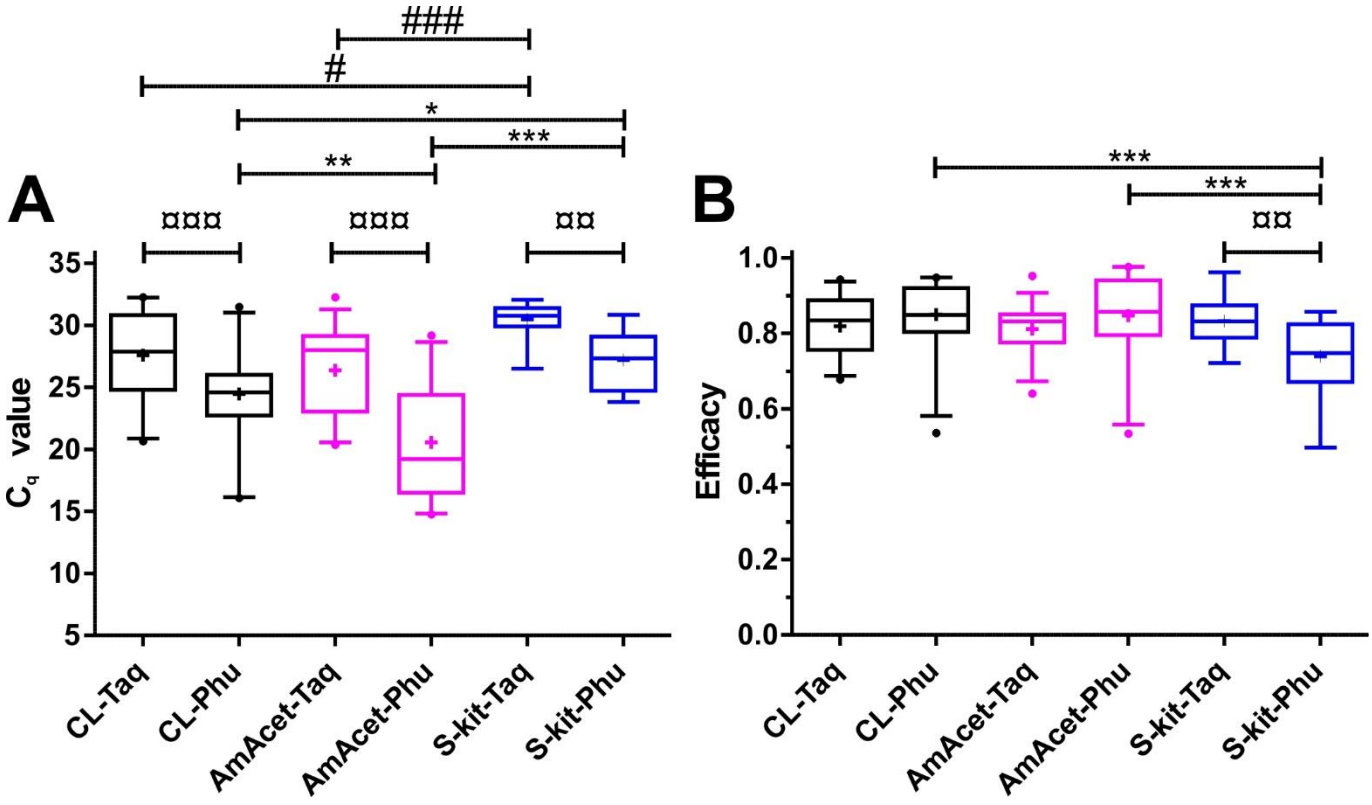

Figure 5. Comparison of cycle of quantification (C_q value) (A) and PCR efficacies (B) between different DNA preparation methods and amplification protocols for a cox-2 partial PCR. Template DNA was obtained as a crude lysate (CL) by simple boiling of mechanically cracked larvae, by proteinase K digestion followed by precipitation with ammonium acetate (AmAcet) or using a soil DNA isolation kit (S-kit). Amplification was performed as real-time PCR using either the FIREPol® Taq (Taq) or the Phusion® (Phu) DNA polymerase protocols. Boxplots show medians with interquartile ranges and whiskers represent 5 and 95% quantiles. Outliers are indicated by dots. The mean of all values is shown as cross. Paired t-tests for the same template DNA using either Taq or Phusion® polymerase protocols did not reveal any significant differences. Comparison between different DNA preparation methods using the same amplification protocol were conducted using One-way ANOVAs. # were used to indicate differences between DNA preparation methods for the Taq polymerase protocol, while * indicate differences between preparation methods for the Phusion® protocol. ¤ was used to indicate differences between FIREPol® Taq and Phusion® in paired analyses. ¤,*,#, $p < 0.05$; ¤¤,**,##, $p < 0.01$; ¤¤¤,***,###, $p < 0.001$

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

3.3. Specificity of Tunga penetrans Partial ITS 2 PCR

Tunga penetrans and *T. trimamillata* plasmids based on their published ITS-2 gene sequences were used to evaluate the specificity of the *T. penetrans* specific primer pair using the Phusion® DNA polymerase real-time PCR protocol. Independent of the amount of template DNA (10^1-10^5 copies per reaction), amplification was observed for all three replicates with *T. penetrans* plasmid as template while all replicates with *T. trimamillata* ITS-2 as template were negative. Moreover, no cross reaction with cat flea DNA prepared with the CL protocol was observed (Table 3).

Table 3. Specificity of the *Tunga-penetrans* specific PCR. Different amounts of plasmids containing the ITS-2 region of *T. penetrans* and *Tunga trimamillata* and genomic DNA from *Ctenocephalides felis* larvae were used as template for the *T. penetrans*-specific real-time PCR.

Target quantity (copy numbers)	**1×10^{5} [a]**	**1×10^{3} [a]**	**1×10^{1} [a]**
	C_q value (mean [range])	**C_q value (mean [range])**	**C_q value (mean [range])**
Target species			
T. penetrans	22.49 [2.946]	25.81 [5.239]	28.46 [3.198]
T. trimamillata	n.a.	n.a.	n.a.
C. felis (genomic DNA)[b]	n.a.	n.a.	n.a.

n.a., not available.

[a]n = 3

[b]Copy number unknown.

4. Discussion

The aim of this study was to establish DNA preparation and PCR protocols that allow low-cost, high throughput molecular processing of large numbers of *Tunga* off-host stages in preparation for extensive ecological and epidemiological tungiasis risk factor studies. Six protocols including all combinations of three DNA preparation methods with two PCR enzymes were compared to identify a reliable and low-cost method for the identification of *T. penetrans* with a particular focus on off-host stages such as larvae, for which no morphological keys are currently available. The *T. penetrans*-specific partial ITS-2 primer pair is the first one designed to discriminate *T. penetrans* from other flea species including *T. trimamillata* and can in the future be used to identify juvenile off-host and adult on-host stages of *T. penetrans* in field samples. This approach can then be used to document the morphology of all stages of the flea species.

Following DNA preparation, PCR assays targeting cox-2 gene were initially performed using a conventional hot-start Taq polymerase (GoTaq) and Phusion® polymerases. However, GoTaq, the by far cheapest of all polymerases used here, was not further evaluated due to an unsatisfactory success rate compared to Phusion®. Instead, FIREPol® Taq and Phusion® were compared in all subsequent experiments. Both of these polymerases have a higher processivity than conventional Taq and were chosen to compensate for potential presence of PCR inhibitors in soil samples. An alternative approach is the removal of such inhibitors during the DNA preparation process using commercially available kits which are optimized to extract DNA from microorganisms in soil samples. Due to an additional purification step, they are considerably more expensive than DNA extraction kits used for tissue samples.

Among the three DNA preparation methods, the S-kit turned out to be the by far most expensive and showing the poorest results in terms of success rate for PCR, highest

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 November 2022 doi:10.20944/preprints202211.0203.v1

C_q values and lowest PCR efficacy. Differences between the two low cost methods-AmAcet and CL – were negligible although there was a tendency for lower C_q values and higher PCR success rates for the AmAcet approach compared with the crude lysate prepared by mechanical disruption and boiling in the CL protocol. However, the CL protocol has the advantage that it only requires a water bath or heat block and no further laboratory equipment such as a centrifuge. It also requires only minimal handling of samples which reduces the risk of contamination of samples. The poor performance of the S-kit in comparison to the other methods was unexpected. A likely explanation might be the very small amount of starting material. Soil DNA kits are optimised for a defined amount of soil, in this case 500 mg, however, *T. penetrans* larvae are less than half of the size of a *C. felis* larva and even unfed adult *C. felis* have a weight below 0.5 mg [34]. With such a small amount of starting material, the S-kit protocol might result in a sub-optimal DNA yield.

Differences between the types of polymerases were also small. If significant differences in amplification efficiency were found, they were most often observed for the larger cox-2 PCR product. On one hand, the number of successful sample amplifications of the cox-2 PCR was significantly higher for FIREPol® Taq than for Phusion® when the AmAcet protocol was used for DNA preparation as compared to the other two DNA preparation methods. On the other hand, C_q values were significantly lower for Phusion® than for FIREPol® Taq with all three DNA preparation methods. Since this was not accompanied by higher PCR efficacies as determined using LinRegPCR, the significantly lower C_q values in the paired data analyses using the same template DNA suggests that other differences in the PCR protocols contributed to this effect. One possible explanation is a brighter fluorescence signal of double stranded DNA in the Phusion®-based assay. Even though both assays use EvaGreen as double-stranded DNA-specific dye, the differences in EvaGreen concentration cannot be excluded as a cause for the different results since the EvaGreen concentration is not provided by the supplier in the product information of the FIREPol® EvaGreen qPCR Supermix. Another important difference between both PCR reaction mixtures is the presence of dUTP in the FIREPol® EvaGreen qPCR Supermix which is known to be incompatible with some PCR enzymes. However, since no significant differences were observed in efficacy in the exponential amplification phase presence of dUTP is an unlikely explanation.

The use of the soil kit leads to costs that are approximately six-fold higher than that of AmAcet approach and 20-fold higher than that of CL approach. Both AmAcet and CL combined with either of the polymerase deliver acceptable results and the decision on the method can be based on the price which clearly favours the combination CL with Phusion® polymerase.

The choice of primer pairs for the present study was guided by two different considerations. The cox-2 primer pair has been frequently used in studies on the phylogeny of Siphonaptera and is therefore well known to amplify partial mitochondrial DNA fragments from many flea species [31,32]. This also means that there is a considerable number of flea cox-2 sequences available in GenBank and this PCR can therefore be used in future projects to identify larvae from species that were negative in the *T. penetrans*-specific PCR leading to an improvement of our knowledge regarding the specificity of the partial *T. penetrans* ITS-2 PCR over time. The *T. penetrans*-specific PCR can be used in ongoing and future projects to rapidly identify larvae collected in households or stables to identify sites of *T. penetrans* development and transmission. It can also be used to replace morphological identification of adult *Tunga* spp. although morphological identification is possible for them using a published key [34].

In South America there are three confirmed synanthropic *Tunga* species infecting humans, companion animals and/or livestock, i.e. *T. penetrans, T. trimamillata* (both zoonotic) and *Tunga hexalobulata* (only known to infect cattle so far) [2,35] as well as *Tunga caecata* infecting synantropic rats [3]. In this context, further evaluation of the specificity of the *T. penetrans*-specific PCR will be required. In Africa, only *T. penetrans* is endemic and it will be sufficient to confirm specificity of the PCR by sequencing the PCR product for a small subset of positive samples to confirm identity in future field studies.

 doi:10.20944/preprints202211.0203.v1

In conclusion, the present study has evaluated a set of DNA preparation/PCR protocols and identified low cost approaches to identify flea larvae from soil samples. The approximately 20-fold decrease in costs compared to the use of a soil DNA isolation kit is highly relevant for resource poor settings and the developed low-cost protocols will allow to screen much higher numbers of samples collected in field studies. While *T. penetrans* can be directly detected using a species-specific PCR or any flea larvae by a cox-2 PCR followed by sequencing, applying these PCRs in future field studies will allow to further characterise their sensitivity and specificity. The same approach can in the future also be adapted to be used for other arthropods from PCR-inhibitor rich matrices such as soil or faeces.

Author Contributions: Conceptualization, Billy Amugune, Abneel Matharu, Lynne Elson and Ulrike Fillinger; Data curation, Billy Amugune, Abneel Matharu and Ulrike Fillinger; Formal analysis, Billy Amugune and Abneel Matharu; Funding acquisition, Ulrike Fillinger; Investigation, Billy Amugune, Abneel Matharu and Paul Ouma; Methodology, Billy Amugune, Abneel Matharu, Paul Ouma, Francis Mutebi and Ulrike Fillinger; Project administration, Ulrike Fillinger; Resources, Ulrike Fillinger; Supervision, Ulrike Fillinger; Validation, Billy Amugune and Abneel Matharu; Visualization, Billy Amugune and Abneel Matharu; Writing – original draft, Billy Amugune and Abneel Matharu; Writing – review & editing, Billy Amugune, Abneel Matharu, Paul Ouma, Francis Mutebi, Lynne Elson and Ulrike Fillinger.

Funding: This research was funded by the German Research Foundation (DFG) through the project "Tungiasis in East-Africa - an interdisciplinary approach to understand the interactions between parasite and host" (project number 405027164; KR 2245/7-1) granted to Jürgen Krücken, Amina Abubaker, Ulrike Fillinger and Charles Waiswa. Additional support was provided from institutional funding for the International Centre of Insect Physiology and Ecology (*icipe*) from the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Federal Democratic Republic of Ethiopia; and the Government of the Republic of Kenya. The funders had no role in study design, decision to publish, or preparation of the manuscript.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Kenya Medical Research Institute's Scientific Ethics Review Unit (KEMRI-SERU; protocol no. 644; initial approval 13.12.2018, renewed annually)

Data Availability Statement: Data is contained within the article or supplementary material.

Acknowledgments: We are grateful for the approval from household residents to allow us sampling of floors. We thank icipe staff Rose Ongole for technical assistance, Ian Okello and Joshua Otiang' for helping in sample collection, and Ibrahim Kiche for logistic support. We also thank Peggy Hoffmann-Köhler (Freie Universität Berlin) for providing cat flea larvae used in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Feldmeier, H.; Keysers, A. Tungiasis - A Janus-Faced Parasitic Skin Disease. *Travel Med. Infect. Dis.* **2013**, *11*, 357–365, doi:10.1016/j.tmaid.2013.10.001.
2. Mutebi, F.; Krücken, J.; Feldmeier, H.; Von Samson-Himmelstjerna, G. Clinical Implications and Treatment Options of Tungiasis in Domestic Animals. *Parasitol. Res.* **2021**, *120*, 4113–4123, doi:https://doi.org/10.1007/s00436-021-07121-y.
3. Harvey, T.V.; Linardi, P.M.; Carlos, R.S.A.; Heukelbach, J. Tungiasis in Domestic, Wild, and Synanthropic Animals in Brazil. *Acta Trop.* **2021**, *222*, 106068, doi:10.1016/J.ACTATROPICA.2021.106068.
4. Feldmeier, H.; Heukelbach, J.; Ugbomoiko, U.S.; Sentongo, E.; Mbabazi, P.; von Samson-Himmelstjerna, G.; Krantz, I. Tungiasis—A Neglected Disease with Many Challenges for Global Public Health. *PLoS Negl. Trop. Dis.* **2014**, *8*, doi:10.1371/journal.pntd.0003133.

5. Villagrana, S.M.N.; Santisteban, A.G.N. Tungiasis: A Highly Neglected Disease among Neglected Diseases. Case Series from Nduta Refugee Camp (Tanzania). *Oxford Med. Case Reports* **2019**, *2019*, 267–270, doi:10.1093/omcr/omz049.
6. Miller, H.; Ocampo, J.; Ayala, A.; Trujillo, J.; Feldmeier, H. Very Severe Tungiasis in Amerindians in the Amazon Lowland of Colombia: A Case Series. *PLoS Negl. Trop. Dis.* 2019, *13*.
7. Mutebi, F.; Krücken, J.; Feldmeier, H.; Waiswa, C.; Mencke, N.; Von Samson-Himmelstjerna, G. Tungiasis-Associated Morbidity in Pigs and Dogs in Endemic Villages of Uganda. *Parasites and Vectors* **2016**, *9*, doi:10.1186/s13071-016-1320-0.
8. Mutebi, F.; Krücken, J.; Mencke, N.; Feldmeier, H.; G., V.S.-H.; Waiswa, C. Two Severe Cases of Tungiasis in Goat Kids in Uganda. *J. Insect Sci.* **2016**, *16*, 3–5, doi:10.1093/jisesa/iew016.
9. Mutebi, F.; Krücken, J.; Feldmeier, H.; Waiswa, C.; Mencke, N.; Eneku, W.; von Samson-Himmelstjerna, G. High Intensity of Tunga Penetrans Infection Causing Severe Disease among Pigs in Busoga, South Eastern Uganda. *BMC Vet. Res.* **2017**, *13*, 1–9, doi:10.1186/s12917-017-1127-z.
10. Harvey, T.V.; dos Santos Freire, Z.; dos Santos, K.C.; de Jesus, A.V.; Guedes, P.E.B.; da Paixão Sevá, A.; de Almeida Borges, F.; Carlos, R.S.A. Clinical and Macroscopic Morphological Features of Canine Tungiasis. *Parasitol. Res.* **2021**, *120*, 807–818, doi:10.1007/s00436-020-07013-7.
11. Bitam, I.; Dittmar, K.; Parola, P.; Whiting, M.F.; Raoult, D. Fleas and Flea-Borne Diseases. *Int. J. Infect. Dis.* **2010**, *14*, e667–e676, doi:10.1016/j.ijid.2009.11.011.
12. Durden, L.A.; Hinkle, N.C. Fleas (Siphonaptera). *Med. Vet. Entomol.* **2019**, 145–169, doi:10.1016/B978-0-12-814043-7.00010-8.
13. Hsu, M.H.; Hsu, Y.C.; Wu, W.J. Consumption of Flea Faeces and Eggs by Larvae of the Cat Flea, Ctenocephalides Felis. *Med. Vet. Entomol.* **2002**, *16*, 445–447, doi:10.1046/j.1365-2915.2002.00388.x.
14. Poulin, R.; Krasnov, B.R.; Shenbrot, G.I.; Mouillot, D.; Khokhlova, I.S. Evolution of Host Specificity in Fleas: Is It Directional and Irreversible? *Int. J. Parasitol.* **2006**, *36*, 185–191, doi:10.1016/J.IJPARA.2005.09.017.
15. Coates, S.J.; Thomas, C.; Chosidow, O.; Engelman, D.; Chang, A.Y. Ectoparasites. *J. Am. Acad. Dermatol.* **2020**, *82*, 551–569, doi:10.1016/j.jaad.2019.05.110.
16. Meng, H.; Xu, S.; Yu, Z.; Li, N.; Wang, R.; Gao, X.; Yang, X.; Liu, J. Abundance and Seasonal Activity of Haemaphysalis Concinna (Acari: Ixodidae) at the Border between China and Russia in Northern Inner Mongolia, China. *Parasites and Vectors* **2016**, *9*, 1–7, doi:10.1186/s13071-015-1291-6.
17. Krasnov, B.R.; Khokhlova, I.S.; Fielden, L.J.; Burdelova, N. V. Development Rates of Two Xenopsylla Flea Species in Relation to Air Temperature and Humidity. *Med. Vet. Entomol.* **2001**, *15*, 249–258, doi:10.1046/j.0269-283X.2001.00295.x.
18. Krasnov, B.R.; Khokhlova, I.S.; Fielden, L.J.; Burdelova, N.V. Effect of Air Temperature and Humidity on the Survival of Pre-Imaginal Stages of Two Flea Species (Siphonaptera: Pulicidae). *J. Med. Entomol.* **2001**, *38*, 629–637, doi:10.1603/0022-2585-38.5.629.
19. Rust, M.K. Recent Advancements in the Control of Cat Fleas. *Insects* **2020**, *11*, 1–17, doi:10.3390/insects11100668.
20. Hoeppli, R. Early References to the Occurrence of Tunga Penetrans in Tropical Africa. *Acta Trop.* **1963**, *20*, 143–153.
21. Deka, M.A. Mapping the Geographic Distribution of Tungiasis in Sub-Saharan Africa. *Trop. Med. Infect. Dis.* **2020**, *5*, 1–20, doi:10.3390/TROPICALMED5030122.
22. Matheson, C.D.; Gurney, C.; Esau, N.; Lehto, R. Assessing PCR Inhibition from Humic Substances. *Open Enzym. Inhib. J.* **2010**, *3*, 38–45, doi:10.2174/1874940201003010038.
23. Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR Inhibitors - Occurrence, Properties and Removal. *J. Appl. Microbiol.* **2012**, *113*, 1014–1026, doi:10.1111/j.1365-2672.2012.05384.x.
24. Moreira, D. Efficient Removal of PCR Inhibitors Using Agarose-Embedded DNA Preparations. *Nucleic Acids Res.* **1998**, *26*, 3309–3310, doi:10.1093/nar/26.13.3309.
25. McInerney, P.; Adams, P.; Hadi, M.Z. Error Rate Comparison during Polymerase Chain Reaction by DNA Polymerase. *Mol. Biol. Int.* **2014**, *2014*, 1–8, doi:10.1155/2014/287430.
26. Miura, M.; Tanigawa, C.; Fujii, Y.; Kaneko, S. Comparação de Seis Polimerases de DNA Disponíveis Comercialmente Para o

PCR Direto. *Rev. Inst. Med. Trop. Sao Paulo* **2013**, *55*, 401–406, doi:10.1590/S0036-46652013000600005.

27. Trombley Hall, A.; McKay Zovanyi, A.; Christensen, D.R.; Koehler, J.W.; Devins Minogue, T. Evaluation of Inhibitor-Resistant Real-Time PCR Methods for Diagnostics in Clinical and Environmental Samples. *PLoS One* **2013**, *8*, 1–8, doi:10.1371/journal.pone.0073845.
28. Demeler, J.; Ramünke, S.; Wolken, S.; Ianiello, D.; Rinaldi, L.; Gahutu, J.B.; Cringoli, G.; von Samson-Himmelstjerna, G.; Krücken, J. Discrimination of Gastrointestinal Nematode Eggs from Crude Fecal Egg Preparations by Inhibitor-Resistant Conventional and Real-Time PCR. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0061285.
29. González, G.; Barberena-arias, M.F.; Huang, W. Chapter 19 Sampling Methods for Soil and Litter Fauna. **2020**, doi:10.1007/978-3-030-53226-0.
30. Mutebi, F.; Krücken, J.; Feldmeier, H.; Waiswa, C.; Mencke, N.; Sentongo, E.; von Samson-Himmelstjerna, G. Animal Reservoirs of Zoonotic Tungiasis in Endemic Rural Villages of Uganda. *PLoS Negl. Trop. Dis.* **2015**, *9*, doi:10.1371/journal.pntd.0004126.
31. Whiting, M.F. Mecoptera Is Paraphyletic: Multiple Genes and Phylogeny of Mecoptera and Siphonaptera. *Zool. Scr.* **2002**, *31*, 93–104, doi:10.1046/j.0300-3256.2001.00095.x.
32. Hornok, S.; Beck, R.; Farkas, R.; Grima, A.; Otranto, D.; Kontschán, J.; Takács, N.; Horváth, G.; Szoke, K.; Szekeres, S.; et al. High Mitochondrial Sequence Divergence in Synanthropic Flea Species (Insecta: Siphonaptera) from Europe and the Mediterranean. *Parasites and Vectors* **2018**, *11*, 1–11, doi:10.1186/s13071-018-2798-4.
33. Luchetti, A.; Mantovani, B.; Pampiglione, S.; Trentini, M. Molecular Characterization of Tunga Trimamillata and T. Penetrans (Insecta, Siphonaptera, Tungidae): Taxonomy and Genetic Variability. *Parasite* **2005**, *12*, 123–129, doi:10.1051/parasite/2005122123.
34. Dryden, M.W.; Rust, M.K. The Cat Flea: Biology, Ecology and Control. *Vet. Parasitol.* **1994**, *52*, 1–19, doi:https://doi.org/10.1016/0304-4017(94)90031-0.
35. De Avelar, D.M.; Facury Filho, E.J.; Linardi, P.M. A New Species of Tunga (Siphonaptera: Tungidae) Parasitizing Cattle from Brazil. *J. Med. Entomol.* **2013**, *50*, 679–684, doi:10.1603/me12221.