Tropical Medicine and Infectious Disease

One Health and Neglected Tropical Diseases

Edited by

Claire J. Standley, Jared Bakuza and Jennifer K. Peterson

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Cover image courtesy of Jennifer K. Peterson.

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About the Editors

Claire J. Standley is an Assistant Research Professor at Georgetown University's Center for Global Health Science and Security, with a primary faculty appointment in the Department of Microbiology & Immunology. She also holds an affiliation with the Heidelberg Institute of Global Health in Germany. Prior to joining Georgetown University, Dr. Standley held positions at the George Washington University, the U.S. Department of State, and Princeton University. Dr. Standley received her Ph.D. in Genetics from the University of Nottingham (joint program with the Natural History Museum, London) and MSc and BA degrees from the University of Oxford and University of Cambridge, respectively.

Jared Bakuza completed his BS in Zoology/Wildlife Ecology and MS in Applied Zoology (Parasitology), both from University of Dar es Salaam, and he received his Ph.D. from the University of Glasgow, UK after writing a thesis on the ecology of *Schistosoma mansoni* infection in sympatric human and non-human primates in the Gombe ecosystem, Tanzania. From 2001 to 2008, Dr. Bakuza worked on baboon research and conservation and was later manager of the chimpanzee health monitoring project in Gombe National Park, Tanzania. Towards the end of 2008, he joined the University of Dar es Salaam, where has been teaching and supervising students in biological and biomedical sciences. Dr. Bakuza's recent research work has focused on studying the impact of sanitation and hygienic practices on parasitic infections.

Jennifer K. Peterson obtained her Ph.D. in Ecology and Evolutionary Biology from Princeton University in 2015. Following completion of her graduate work, Dr. Peterson carried out postdoctoral studies in disease modeling at Princeton University, as part of the Gates-funded NTD modeling group. She then shifted gears to carry out postdoctoral work in disease ecology and epidemiology with the University of Pennsylvania, stationed at the Zoonotic Disease Research Lab in Arequipa, Peru. Dr. Peterson's work focuses on parasite-host-vector interactions across scales, and she specializes in Chagas disease, eco-epidemiology, and global health, with a focus on Latin America.

Tropical Medicine and Infectious Disease

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Editorial

One Health and Neglected Tropical Diseases—Multisectoral Solutions to Endemic Challenges

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One Health is defined as an approach to achieve better health outcomes for humans, animals, and the environment through collaborative and interdisciplinary efforts. Increasingly, the One Health framework is being applied to the management, control, and even elimination of neglected tropical diseases (NTDs). NTDs are a set of debilitating and often chronic infectious diseases that, collectively, affect more than one billion people in almost 150 countries, with disproportionate impact on the extremely poor [1,2]. In this Special Issue, we present a diverse body of work united under the One Health ideology and a desire to mitigate the devastating effects of NTDs. The numerous diseases, methodologies, and landscapes presented highlight the interconnected and increasingly overlapping existence of humans, animals, and their pathogens.

The global scope of the papers demonstrates the scale at which NTDs affect daily life. From Latin America and the Caribbean to South Asia and sub-Saharan Africa, NTDs are an ever-present reality for far too many people. The articles also highlight that NTDs are both an urban and a rural problem, and the frequency with which they cause disease in animals and persist in zoonotic reservoirs exemplifies the expansive utility of the One Health approach across diverse disease systems.

Broadly, the works presented touch upon three common themes. First, the nature of zoonotic NTDs is such that eradication is rarely an option; animal reservoirs provide a persistent source of re-infection, leaving local elimination in human populations as the most feasible public health goal. One Health coordination across multiple sectors and disciplines is therefore required to achieve such goals, which necessitates interrupting ongoing transmission, diagnosing and treating current cases in both humans and animals, and preventing future transmission scenarios from re-emerging via human practices that facilitate animal-human contact and/or human-vector contact. The "how" is of course much more complex, and it varies by disease. For example, in Zambia, Mulenga et al. [3] describe that, while tsetse fly management efforts have focused attention on areas of high livestock production, the lack of integration with human or wildlife considerations has resulted in decreased surveillance of wildlife protection areas and game parks, despite potential risk to local communities and international visitors. In some contexts, ethical considerations may also be involved. For example, in their review of rabies as a historical public health concern in India, Radhakrishnan and colleagues [4] note conflicting perspectives on the importance of animal welfare with respect to management of feral dogs, a major source of human rabies in the country. Finally, the work presented in Boyce et al. [5] demonstrates how a lack of holistic understanding about transmission pathways can complicate control or prevention of a disease. In Chad, increasing numbers of

Guinea worm disease cases detected in dogs pose a major obstacle for elimination of the disease in humans, although the exact pathways by which dogs are exposed and/or contribute to onward transmission is still under debate.

Second, some of the works in this issue touch upon bureaucratic or semantic obstacles standing in the way of countries and organizations receiving international support for combatting certain diseases. Support from global health agencies such as the World Health Organization (WHO) is often critical for disease control, but to receive such assistance, the country or disease in question must undergo what can often, regrettably, be a political process to receive formal designation or recognition that a disease is "endemic", "neglected", or "zoonotic". Such is the case with Chagas disease in Trinidad and Tobago, where despite mounting evidence that *Trypanosoma cruzi* transmission to humans occurs , the nation is not yet formally recognized as 'Chagas endemic' by WHO [6], and as such, does not receive support to combat the disease. Similarly, toxocariasis and giardiasis are not officially recognized as NTDs by WHO, despite having many of the same characteristics and impacts, not to mention the potential benefits for increased scientific attention, funding, and prioritization that could come with being grouped with the NTDs [7,8]. Finally, as mentioned above, existing eradication approaches for Guinea worm disease have been focused solely on humans; recognition of its zoonotic nature is required to ensure future success [5]. We hope that these studies encourage us to re-examine the power wielded by semantics in public health, and also to think about how we might loosen the grip that official designations such as "NTD", "endemic", and "zoonotic" have on global health resource allocation. These words are meant to facilitate and direct global health efforts, but, sadly, in these cases, may function as exclusionary devices.

Finally, several of the articles in this issue provide exciting opportunities for integrating new strategic and methodological approaches to enhance One Health approaches. For instance, Chowdhury et al. [9] focus on improving point-of-need diagnostics with new molecular methods for rapid detection of kala-azar leishmaniasis in resource-limited settings. Yeh et al. [10] argue that genomic technologies may be able to track changes in distribution of NTDs such as schistosomiasis in the face of climate change, as well as elucidate shifting parasite-vector dynamics. "Mainstreaming" and "integration" are key buzzwords in NTD control, recognizing the challenges related to sustainable control of these endemic and persistent pathogens in the decades ahead. As Archer et al. [8] highlight, there may be strong scientific rationales for aligning efforts between waterborne parasitic infections such as schistosomiasis and giardiasis, especially when there are substantial knowledge gaps surrounding the distribution, burden, and zoonotic potential of the latter.

To this end, the articles in this Special Issue present a compelling and comprehensive snapshot of the myriad challenges facing NTD control, highlighting the complex ways in which different pathogens impact—and are impacted by—human, animal and environmental health. Importantly, they also showcase opportunities and innovations for harnessing the collaborative and holistic potential of One Health to reduce the impact of some of the world's most widespread and burdensome diseases.

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

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Article

Preliminary Characterization of Triatomine Bug Blood Meals on the Island of Trinidad Reveals Opportunistic Feeding Behavior on Both Human and Animal Hosts

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Abstract: Chagas disease is a neglected tropical disease caused by infection with *Trypanosoma cruzi*. The parasite is endemic to the Americas, including the Caribbean, where it is vectored by triatomine bugs. Although Chagas disease is not considered a public health concern in the Caribbean islands, studies in Trinidad have found *T. cruzi*-seropositive humans and *T. cruzi*-infected triatomine bugs. However, little is known about triatomine bug host preferences in Trinidad, making it difficult to evaluate local risk of vector-borne *T. cruzi* transmission to humans. To investigate this question, we collected triatomine bugs in Trinidad and diagnosed each one for *T. cruzi* infection (microscopy and PCR). We then carried out a blood meal analysis using DNA extracted from each bug (PCR and sequencing). Fifty-five adult bugs (54 *Panstrongylus geniculatus* and one *Rhodnius pictipes*) were collected from five of 21 sample sites. All successful collection sites were residential. Forty-six out of the 55 bugs (83.6%) were infected with *T. cruzi*. Fifty-three blood meal hosts were successfully analyzed (one per bug), which consisted of wild birds (7% of all blood meals), wild mammals (17%), chickens (19%), and humans (57%). Of the 30 bugs with human blood meals, 26 (87%) were from bugs infected with *T. cruzi*. Although preliminary, our results align with previous work in which *P. geniculatus* in Trinidad had high levels of *T. cruzi* infection. Furthermore, our findings suggest that *P. geniculatus* moves between human and animal environments in Trinidad, feeding opportunistically on a wide range of species. Our findings highlight a critical need for further studies of Chagas disease in Trinidad in order to estimate the public health risk and implement necessary preventative and control measures.

Keywords: chagas disease; *Trypanosoma cruzi*; triatomine bugs; *Panstrongylus geniculatus*; *Rhodnius pictipes*; Trinidad and Tobago; West Indies; vector host-feeding preferences; blood meal analysis

1. Introduction

Chagas disease (American trypanosomiasis) is a neglected tropical disease (NTD) caused by infection with the protozoan parasite *Trypanosoma cruzi*. Symptoms of the disease include cardiac,

gastrointestinal, and/or neurological alterations. An estimated six million people are currently infected with *T. cruzi*, with 25 million more at risk [1].

T. cruzi is endemic to the Americas, where it cycles between mammalian hosts and triatomine bug vectors. Vector-borne *T. cruzi* transmission to humans can occur via contact with infected triatomine excrement during or after the bug takes a blood meal, or orally, through ingestion of *T. cruzi*-contaminated food or beverages [2–5]. Food or beverage contamination with *T. cruzi* can occur in a number of ways, but most commonly an infected bug either falls into or defecates on the food. Other human *T. cruzi* infection routes include contaminated blood transfusions or organ transplants, transplacental transmission, and laboratory accidents [1].

Triatomine bugs acquire *T. cruzi* infection by taking blood meals from infected mammals in sylvatic or domestic/peridomestic transmission cycles. These transmission cycles can be connected, especially in disturbed or fragmented habitats [6], by triatomines that forage opportunistically in both their sylvatic habitats and nearby residential areas, including human homes [7,8]. Due to the existence of distinct *T. cruzi* transmission cycles, the presence of triatomine bugs in a region does not necessarily mean that the region is Chagas-endemic. Such is the case in the Caribbean islands, where triatomine bugs are widely distributed [9], but believed to be primarily sylvatic, making infrequent contact with humans [9–11]. Although *T. cruzi*-positive serology in humans has been reported in at least three Caribbean islands (Curacao, Jamaica, and Trinidad [9,12–14]), there have been few diagnosed clinical cases. As such, Chagas disease is not recognized by major public or global health organizations as endemic to any Caribbean island [10,15].

Of particular interest for Chagas disease in the Caribbean is the island of Trinidad, of the dual-island Republic of Trinidad and Tobago. Trinidad has geological origins on the continental shelf of South America [16], and shares much of its flora and fauna with Chagas-endemic, neighboring Venezuela (located just eight miles west of Trinidad, Figure 1). Included in the fauna found in both Trinidad and Venezuela are six triatomine bug species: *Eratyrus mucronatus*, *Microtriatoma trinidadensis*, *Rhodnius pictipes*, *Panstrongylus geniculatus*, *Panstrongylus rufotuberculatus*, and *Triatoma rubrofasciata* [17–19]. All species are competent vectors of *T. cruzi* [11], but *P. geniculatus* is believed to be the most abundant [17], and of the highest epidemiological importance [3,20–24].

Although there are only a few studies of Chagas disease in Trinidad, they provide compelling evidence that *T. cruzi* may not be strictly enzootic on the island. Cardiologist Boris Fistein first suspected that Chagas disease was endemic to the island in the early 1960s, when he observed the clinical picture of the disease in several patients with congestive heart failure not attributable to common causes [12,13]. Fistein found that some of these patients were seropositive for *T. cruzi* [12,13,25], and a follow-up study of the vector yielded 79 triatomine bugs collected in and around the patients' homes. Of 69 bugs examined, 35 (50.72%) were infected with *T. cruzi* [25]. A later study of sera collected from venereal disease clinics and antenatal clinics (meant to represent the general population) found a *T. cruzi* seroprevalence of 0.45% [12]. Several years later, a larger study of triatomines in Trinidad yielded the same species infected with *T. cruzi* distributed widely throughout the island [17]. Finally, in a 1992 serological survey of 192 cardiac patients in south Trinidad, 72 (37.50%) tested positive for *T. cruzi* antibodies [26]. The authors report that 49 of the 72 patients (68.06%) had *T. cruzi* trypomastigotes in peripheral blood, although this number seems unusually high.

While these findings provide mounting evidence of a vector-borne Chagas disease transmission cycle existing in Trinidad, there are no known published data demonstrating the species on which *T. cruzi*-infected triatomine bugs are feeding, other than a single wild rat found infected with *T. cruzi* in 1963 [27]. Identification of the host species with which triatomines come into contact by taking a blood meal can give us a sense of the frequency with which the bugs invade domestic environments, and in turn, the risk of vector-borne Chagas disease. For example, a diet of primarily wild animal hosts would suggest that there is little risk of the disease on the island, while a diet involving domestic animals and/or humans would imply that Chagas disease may be of local epidemiologic importance. As such, we asked, from which species are *T. cruzi*-infected triatomines in Trinidad taking blood meals?

We aimed to use our findings to further characterize the current *T. cruzi* transmission cycle in Trinidad, and in turn, better understand the risk of Chagas disease transmission on the island.

Figure 1. Location of Trinidad and Tobago relative to South America, Venezuela, and other Caribbean islands. Map created with the assistance of T. W. Shawa, Princeton Geographic Information Systems Librarian.

2. Materials and Methods

2.1. Overview

Triatomine bugs were sampled on the island of Trinidad between May and August, 2016. Bugs were collected using mouse-baited traps placed near animal resting/nesting sites and artificial light sources (Figure 2), in addition to manual collection when a bug was found resting on a wall or other surface. Public submissions were also accepted. Upon collection, bugs were examined for *T. cruzi* infection before undergoing blood meal analysis to identify a species on which each bug had recently taken a blood meal.

Figure 2. Successful mouse-baited trap. Adult *P. geniculatus* is caught on the adhesive material lining the outside of the trap. The trap is set on a windowsill, near an artificial light on the exterior of a ranger station in the village of Mt. Harris. Photo by J. K. Peterson.

2.2. Animal Use Ethics Statement

The mouse-baited trap protocol used in this study was reviewed and approved by The Institutional Animal Care and Use Committee (IACUC) of Princeton University (protocol # 2065F-16) and St. George's University (approval number 16010-R), and the Campus Ethics Committee at the University of the West Indies (reference number CEC172/04/16).

2.3. Study Sites

A total of 21 sites were sampled across 11 villages concentrated mostly in northern and central Trinidad (site-specific details in Table S1, Supplementary Materials). All sites except one were residential or very close to human disturbance (i.e., residential, farm, logging, alongside a road, etc.). Within each site, particular attention was paid to *Attalea maripa* palms known locally as 'cocorite,' which are known habitat for local fauna. We sampled for one night per location, with repeat sampling of a site occurring after a successful collection.

2.4. Triatomine Bug Collection

We used three methods to collect triatomine bugs: mouse-baited traps, manual trapping, and community collection. Mouse-baited traps (Figure 2, [28]) were placed in animal resting sites, as triatomines tend to live near their food source. These sites included rat nests, opossum nests, bat-infested structures, and 18 *A. maripa* palms (details for each site in Table S1). In residential areas, we placed traps near light sources in building exteriors.

Mouse-baited traps consisted of a 'T-shaped' PVC pipe (3" diameter) covered in Duck® Brand Indoor Heavy Traffic Carpet Tape, which is a double-sided adhesive material (Figure 2). A Swiss albino mouse was placed inside the trap just before use, along with shredded paper for bedding and a piece of potato or fruit that served as a food and water source. Trap openings were closed by a two-inch section of PVC pipe covered in chicken wire and fine mesh. Traps were placed at dusk (5:00 p.m.–8:00 p.m.) and retrieved the next day in the morning (7:00 a.m.–9:00 a.m.). After each use,

the mouse was removed from the trap and returned to its cage. Traps were cleaned and disinfected, and the water source, bedding, and tape were changed before reusing the trap. Each mouse was used in a trap only once per week. Mice were obtained from the laboratory animal rearing facility of the University of the West Indies Veterinary School.

In addition to trapping, we collected bugs opportunistically when found resting on exterior walls near artificial lights. We also accepted public submissions, as described in [29]. Chagas disease information pamphlets with pictures of local bug species were distributed (Figure S1) to those residing near positive collection sites.

2.5. Specimen Processing and Molecular Analyses

All protocols were performed under carefully controlled laboratory conditions. DNA extraction and blood meal analyses were carried out in separate laboratories to minimize cross-contamination risk. Bugs were processed in the Pathology and Parasitology Laboratory at the School of Veterinary Medicine of the University of the West Indies (UWI), St. Augustine, Trinidad. Triatomine species were determined using taxonomic keys and images from Lent and Wygodzinsky [30], Carcavallo et al. [31], and Patterson et al. [32]. *T. cruzi* infection in live bugs was diagnosed through direct microscopy observation (DMO) and staining of bug excrement and stomach contents (Figure 3), while *T. cruzi* infection in dead bugs was diagnosed using PCR. For DMO, 10 µL of Phosphate Buffer Solution (PBS) was mixed with 10 µL of excrement from each bug. A 5 µL aliquot of the solution was examined microscopically at 40× magnification. Trypanosomes were identified by their movement and morphology. Bugs were stored in 70% ethanol (EtOH) for approximately one to three days, until molecular analysis was carried out.

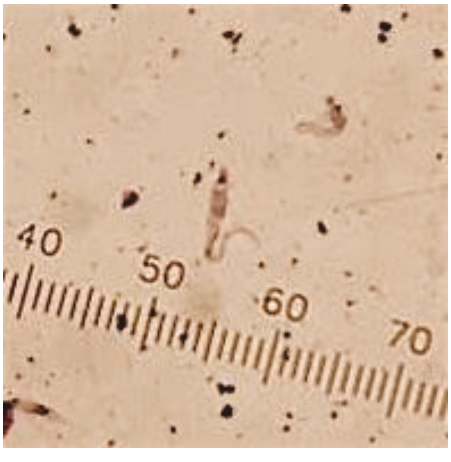

Figure 3. Giemsa stain of *T. cruzi* isolated from *P. geniculatus* collected in this study. Scale shown is in µm. Photo: J. K. Peterson.

DNA was isolated from each bug using the Qiagen DNeasy® Blood and Tissue DNA isolation kit, using a modified protocol for tissue extraction (described in [33]). PCR diagnosis of *T. cruzi* was performed for all bugs using the TcZF/R primer (5′-GCTCTTGCCCACAAGGGTGC-3′ and 5′-CCAAGCAGCGGATAGTTCAGG-3′, [34]), which targets a 182 bp sequence of *T. cruzi* satellite DNA. Following optimization, the final recipe consisted of 1.25 µL of each TcZ-F/R, 1 µL DNA, 21.5 µL dH_2O, and one GE Healthcare illustra PuReTaq Ready-To-Go PCR bead. PCR conditions were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 57 °C for 10 s, 72 °C for 30 s, with a final extension of 72 °C for 7 min, and held at 4 °C. All samples were visualized in a 1.5% agarose gel to confirm amplicon size. To confirm that the amplified PCR product was indeed *T. cruzi*, we extracted

three of the 182 bp bands from the agarose gel following PCR using the Qiagen QIAEX II Gel Extraction Kit, and sent the bands for Sanger sequencing at the Plant-Microbe Genomics Facility at The Ohio State University. We edited sequences with Chromas Lite 2.6.4, and compared them to known sequences in NIH-NCBI GenBank using the Basic Local Alignment Search Tool (BLAST). These analyses confirmed the DNA sequence amplified was from *T. cruzi*.

2.6. Blood Meal Analyses

Blood meal analyses were performed in the Molecular Biology Lab at the St. George's University School of Veterinary Medicine in True Blue, Grenada. We amplified a 360−380 bp fragment of the vertebrate cytochrome b (cytb) gene from the DNA of each bug using the L14841 primer (5′-CCCCTCAGAATGATATTTGTCCTCA-3′) and the H15149 primer (5′-CCATCCAACA TCTCAGCATGATGAAA-3′) [35]. These primers are vertebrate-specific, and are used frequently to preferentially amplify host DNA from blood meals in hematophagous insects [35]. After optimization, the final recipe used was: 1.25 µL of each primer, 2 µL of $MgCl_2$, 3 µL of bug DNA, 17.5 µL of dH_2O, and one GE Healthcare illustra PuReTaq Ready-To-Go PCR bead. All PCRs included appropriate controls. Each sample was run under the following thermal cycling conditions: preheat at 95 °C for 5 min, then 50 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 90 s, followed by 72 °C for 7 min, then hold at 4 °C. All samples were electrophoresed, extracted from gels, sequenced, edited, and compared to known GenBank entries, as described above. The cut off for accepting an identity match in BLAST was ≥90%, and an e-value below 1e−10. (The e-value describes the number of hits one can expect by chance, given database size.) In instances where more than one species had an identity match in BLAST over >90%, the GenBank sequence entry with highest e-value was called. Overlapping, short, or messy sequences were re-run using the same blood meal analysis protocol but with avian-specific and mammal-specific primers that amplified a 508 and 772 bp fragment of the cytb gene, respectively [36]. Sequence data are found in File S1.

3. Results

A total of 55 adult triatomine bugs were collected (54 *Panstrongylus geniculatus* and one *Rhodnius pictipes*) in five of the 11 villages sampled (Table 1). All successful collection spots were in or around human dwellings.

Table 1. Triatomine Bug Collection Site Descriptions with *T. cruzi* Infection Status of Bugs Collected from Each Site, and Human Blood Meals Taken by the Bugs. Only Successful Collection Site Details Are Listed. Details for Every Sample Site Are in Table S1.

Site Name	Location	Site Description	Bugs Collected [1,2]			Human Blood Meals		
			Infected	Uninfected	Total	Infected [3]	Uninfected [3]	Total
Blanchisseuse	Northern coast	Coastal human home adjacent to secondary forest	1 (100%)	0	1	1 (100%)	0	1
Coal Mine	Central range [4]	Scattered human homes adjacent to secondary forest	40 (87%)	6 (13%)	46	22 (92%)	2 (8%)	24
Matura	Northeast coast	Rural human home surrounded by secondary forest	1 (50%)	1 (50%)	2	0	1 (100%)	1
Mt Harris	Central range [4]	Rural neighborhood in close proximity to secondary forest	3 (60%)	2 (40%)	5	2 (67%)	1 (33%)	3
Santa Cruz	Northern range [4]	Laundry area of peri-urban human home	1 (100%)	0	1	1 (100%)	0	1

[1] All bugs collected were *Panstrongylus geniculatus*, except for one *Rhodnius pictipes* male infected with *T. cruzi*. The *R. pictipes* individual was collected manually from the exterior wall of a house in Coal Mine. [2] Sites where just one bug was collected were public submissions, and not sites that were actively sampled. [3] Infection status refers to the bug with the blood meal, not the human.[4] Central and northern ranges are the names of the east-west mountain ranges found in central and northern Trinidad.

No bugs were collected in traps placed in or near animal nesting sites. The majority of bugs (46 of 55; 83.6%) were collected from a single rural area with scattered human dwellings located adjacent to secondary forest. All triatomines collected were in the adult stage; 18 were female and 37 were male.

3.1. *T. cruzi* Infection

T. cruzi DNA was amplified from 46 out of the 55 bugs collected (83.6%). Of these bugs, six were also diagnosed through microscopy (Figure 3; 12 bugs in total were alive upon arrival to the lab). Bugs diagnosed microscopically (in addition to PCR diagnosis) were from Coal Mine (N = 4), Matura (N = 1), and Mt. Harris (N = 1). All successful collection villages (N = 5, Table 1) yielded at least one bug infected with *T. cruzi* (Tables 1 and 2).

Table 2. Blood Meal Species Identified in Triatomine Bugs Collected, and *T. cruzi* Infection Status of the Bug or Bugs Corresponding to Each Species. All Data Are Available in File S1, Including Accession Numbers and Sequences.

Blood Meal Hosts [1]			Triatomine Bugs [2]		Collection Sites [5]	BLAST Results [3]	
Class	Genus/Species	Common Name	Blood Meals [1]	*T. cruzi*-Positive Bugs [4]		% Identity Match	E-Value
Mammalia	*Homo sapiens*	Human	30	26 (87%)	All	99%	1×10^{-58}
	Alouatta seniculus	Red howler monkey	2	2 (100%)	CM, MH	98%	5×10^{-134}
	Dasyprocta leporina	Red-rumped agouti	2	2 (100%)	CM, MT	100%	2×10^{-128}
	Coendou prehensilis	Brazilian porcupine	1	1 (100%)	CM	100%	1×10^{-144}
	Herpestes javanicus	Javan mongoose	1	1 (100%)	CM	100%	8×10^{-146}
	Metachirus nudicaudatus	Brown four-eyed opossum	1	0 (0%)	CM	99%	8×10^{-136}
	Nectomys [6]	Water rat	1	1 (100%)	CM	98%	9×10^{-138}
	Proechimys trinitatis	Trinidad spiny rat	1	0 (0%)	MH	100%	3×10^{-64}
Aves	*Gallus gallus*	Chicken	10	9 (10%)	CM	99%	3×10^{-106}
	Pionus menstruus	Blue-headed parrot	2	1 (50%)	CM	90%	2.5×10^{-77}
	Dryocopus lineatus	Lineated woodpecker	1	1 (100%)	CM	99%	1×10^{-92}
	Pulsatrix perspicillata	Spectacled owl	1	0 (0%)	CM	98%	0 *

[1] One blood meal host was identified per bug. [2] All bugs were *P. geniculatus* except for one *R. pictipes* collected in Coal Mine, which was *T. cruzi* positive and contained a human blood meal. [3] Cut off for accepting an identity match in BLAST was ≥ 90%, and an e-value below 1×10^{-10}. (The e-value describes the number of hits one can expect by chance, given database size.) Average % match and average e-value is shown for species found in more than one blood meal. [4] Positive *T. cruzi* infection in the bug does not mean positive host infection (and birds are not competent for *T. cruzi* infection). [5] CM = Coal Mine, MH = Mount Harris, MT = Matura. Site descriptions in Table 1. [6] BLAST hit corresponded to *Nectomys rattus*, which is not found in Trinidad. The cytb gene sequence for the congeneric species native to Trinidad (*Nectomys palmipes*) is not currently in the Genbank database. * E-values approaching 0 are rounded to 0 in BLAST.

3.2. Blood Meal Analyses

We identified a recent blood meal host species for 53 of the 55 bugs (96.4%; Figure 4, Table 2). Thirty bugs (56.6%) fed on humans, of which 26 (86.7%) were infected with *T. cruzi*, including the single *R. pictipes* individual. The second most common blood meal hosts were chickens (10 bugs; 18.9%), followed by red howler monkeys (2 bugs, 4.0%), red-rumped agoutis (2 bugs, 4.0%), and blue-headed parrots (2 bugs, 4.0%). One blood meal each was identified from a porcupine, mongoose, opossum, water rat, spiny rat, woodpecker, and owl (detailed in Table 2). Overall, nine bugs (17.0%) fed on wild mammals and four bugs (7.5%) fed on wild birds. Each site yielded at least one bug with a human blood meal, and four of the five sites had at least one *T. cruzi*-infected bug with a human blood meal.

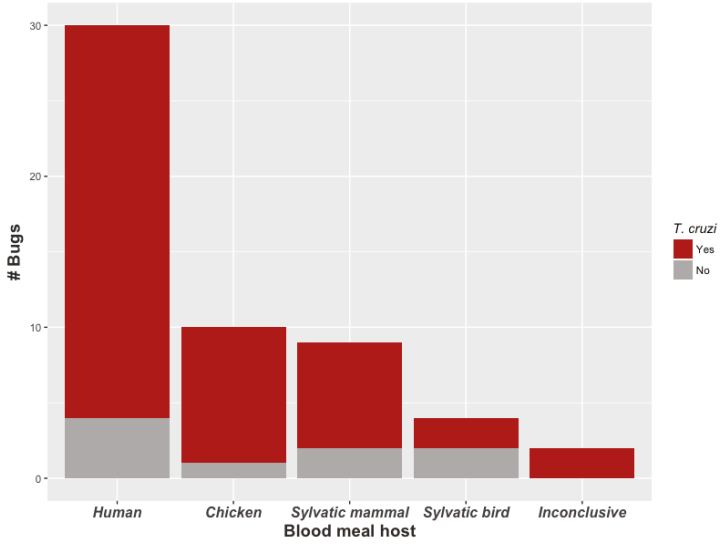

Figure 4. Number of bugs (i.e., blood meals) by blood meal host category and *T. cruzi* infection status of triatomine bug. Data were pooled across sites. One blood meal host was identified per bug. All triatomine species were adult *P. geniculatus* except for one adult male *R. pictipes*, which was infected with *T. cruzi*, and contained a human blood meal. Blood meal host species information is found in Table 2.

4. Discussion

In this study, we aimed to better understand the potential for vector-borne Chagas disease in Trinidad by characterizing the feeding preferences of *T. cruzi* vectors on the island. We found that at least one triatomine bug species on the island, *Panstrongylus geniculatus*, feeds on humans, chickens, and wild animals. While previous studies have found *T. cruzi* infection in humans [12,26] and triatomine bugs [17,25] in Trinidad, our study is the first to demonstrate that the bug feeds opportunistically across a wide range of host species, including humans, on the island.

4.1. P. geniculatus Breeding Habitat Remains Cryptic

All bugs in our study were adults collected from residential areas, despite efforts to sample a wide range of animal resting sites where the bugs might breed (i.e., cocorite palms, opossum nests, bat colonies, etc., Table S1). Still, approximately one-quarter of identified blood meals came from wild animals. Prior studies of *P. geniculatus* in Trinidad found nymphs only in caves and burrows of the nine-banded armadillo (*Dasypus novemcinctus*) [12,17], while in Venezuela, *P. geniculatus* was also associated with the common or black-eared opossum (*Didelphis marsupialis*) [37]. While our blood meal analyses did not reveal either of these host species, we did identify blood meals from several other nesting animals in addition to arboreal species, both of which could provide suitable habitats for the bugs. Thus, it is possible that *P. geniculatus* in Trinidad select breeding sites in a similar manner to how they feed—opportunistically, across a range of species.

Our failure to find juvenile triatomine bugs in residential areas suggests that the bugs forage, but do not breed, in domestic settings. Nonetheless, it important to note that almost one-fifth of identified blood meals in this study were from chickens in residential settings. Although birds are not competent *T. cruzi* hosts, they are a common food source for triatomines, which may in turn amplify *T. cruzi* transmission by increasing carrying capacity of vector populations [38]. Moreover, chicken coops are often found infested with triatomine bugs in other regions, and have even been

used as sentinels for Chagas disease [39]. Thus, it will be of critical importance to sample more widely across residential settings in Trinidad in order to understand the potential for triatomines to breed in domestic settings, especially those with chickens.

4.2. Are Wildlife Population Declines Driving P. geniculatus Host Shifts?

Although we did not find blood meals from nine-banded armadillos in this study, carcasses with *T. cruzi* amastigotes are occasionally brought by hunters to the Veterinary Pathology lab at the University of the West Indies. A larger study would likely yield blood meals from armadillos and possibly *Didelphis* opossums as well, although it is unclear if either species would carry the same importance for *P. geniculatus* as they did 40 years ago (when triatomines were last studied in Trinidad, [17]). Nine-banded armadillos and *Didelphis* opossums are regularly hunted in Trinidad as bushmeat, for which there is high demand; an opossum carcass in Trinidad currently sells for TT$ 300−500 (USD $40−$70). Thus, it is possible that these species are now less abundant, which could be driving *P. geniculatus* into new areas to nest and forage, a phenomenon observed in *P. geniculatus* in the Amazon basin [40,41]. Further studies of wildlife population decline in Trinidad would help identify if reduced wild host species abundance is causing the bugs to leave their nesting sites to feed on humans and domestic animals.

4.3. P. geniculatus: A Cosmopolitan Species of Emerging Importance

P. geniculatus is highly adaptive in both its feeding and habitat preferences. The species is widely distributed in Latin America across a multitude of landscapes [32]. Though historically associated with enzootic *T. cruzi* transmission, the species is increasingly reported in human homes and with human blood meals [21–23]. While once considered to be incapable of domiciliation, the species is now considered to be domiciliated or in the process of domiciliation in several regions [20,21,42], which is reviewed in [43]. Additionally, *P. geniculatus* is no longer limited to forested areas; in the Metropolitan district of Caracas, Venezuela, thousands of *P. geniculatus* were found in both poor and wealthy areas of 31 out of 32 parishes, with nymphs found in 14 of the parishes. The species has also been reported in urban centers in Brazil (Sao Paulo [44] and Rio de Janeiro [45]) and Bolivia (Cochabamba [24]).

4.4. Multiple Paths to T. cruzi Exposure in Trinidad

In addition, it is important to highlight that several different *T. cruzi* transmission routes could pose a risk to humans in Trinidad. The presence of *P. geniculatus* in areas of human use presents a risk for oral transmission of *T. cruzi*. Between 2007 and 2009, there were three oral Chagas disease outbreaks at schools in Venezuela [46,47]. Subsequent analysis implicated *P. geniculatus* contamination of food or drinks in all three outbreaks [23,46,47]. Oral contamination does not require insect domiciliation or colonization of a residence; rather, it can occur merely by triatomines attracted to areas of food preparation by artificial lights. Handling of *T. cruzi*-infected wild animal carcasses by both hunters and researchers in Trinidad presents a *T. cruzi* transmission risk as well [48]. Transmission may also be possible by consuming undercooked, *T. cruzi*-infected bushmeat, although this is yet to be confirmed [49]. Considering the popularity of bushmeat in Trinidad, these transmission routes merit further investigation. Taken together, the multiple possible means of *T. cruzi* exposure in Trinidad suggest that *P. geniculatus* should be taken seriously on the island as a vector species of emerging epidemiologic importance.

4.5. Chagas Disease in Trinidad: Official Endemicity Would Bring Critical Resources

In our experience with community collection, we observed that community members wanted to know more about Chagas disease and its vectors. We were unable to find a local resource to reference, so we created the informational pamphlet (Figure S1 in Supplementary Materials). However, a pamphlet cannot compensate for the resources that are missing due to the country not being officially designated as 'Chagas endemic' by major global health organizations [10,15,50]. With official

Chagas disease endemic status comes numerous resources [50], such as donated medications [51], blood bank monitoring support data coordination [52], and a framework for increasing *T. cruzi* screening in pregnant women [53,54]. Furthermore, endemicity provides the opportunity to participate in subregional intergovernmental Chagas disease control initiatives such as the Southern Cone Initiative (INCOSUR, [55]) and the Initiative for Chagas Disease Control in Central America and Mexico (IPCAM, [56]). Countries in these groups set joint regional goals, and annual meetings to share progress and challenges serve as a source of motivation and accountability. All of the aforementioned resources are available to 'endemic' countries with *T. cruzi* seroprevalences that are likely comparable to Trinidad, such as Costa Rica and Belize, and would be useful in Trinidad, where heart disease was the leading cause of death in 2015 [57].

4.6. Study Limitations

Our results are preliminary, with a small sample size that was limited in geographic distribution and duration. We hope to conduct a larger study in order to define the scope of *T. cruzi* transmission between humans and animals on the island, and determine whether our results are representative of the island as a whole. Additionally, seasonality may have contributed to our failure to find bugs in animal resting sites. Due to logistical constraints, the field work portion of this study took place during the rainy season, when palms, burrows, or other animal nests can be vacated due to humidity or inundation. Indeed, Fistein [12] found that the number of triatomines found inside houses was inversely proportional to both relative humidity and monthly rainfall. Accordingly, follow-up studies should be carried out in the rainy and dry seasons to accurately characterize *P. geniculatus* breeding sites in Trinidad.

Finally, it must be kept in mind that a 'bite' (i.e., blood meal) from a *T. cruzi*-infected triatomine does not guarantee *T. cruzi* transmission, since the parasite is transmitted in vector excrement and not saliva. Thus, our finding of human blood meals in *T. cruzi*-infected bugs does not mean that the bugs transmitted *T. cruzi* to those humans. There is one published study of *P. geniculatus* defecation timing, and the authors found that the bugs do, on average, defecate while feeding [42], which is a key characteristic of epidemiologically important Chagas disease vector species. It is also important to mention that oral transmission is a risk simply with vector presence, regardless of whether or not the bug feeds or defecates on a human host.

5. Conclusions and Future Directions

Although our findings are preliminary, our study is an important first step in furthering our understanding of *T. cruzi* dynamics in Trinidad. The results of our blood meal analysis suggest that *T. cruzi* in Trinidad may not be strictly enzootic, but rather could be a parasite of humans, domestic animals, and wild animals. Our findings support those of prior studies suggesting that Chagas disease in Trinidad needs to be more comprehensively studied, and perhaps re-considered in terms of its public health importance on the island.

Future studies would benefit from taking a One Health approach entailing multidisciplinary research and outreach work focused on interactions between humans, domestic and wild animal populations, triatomine bugs, and the environment. Open questions include triatomine bug seasonality, regional variation, breeding habitats, and juvenile *T. cruzi* infection rates. Identifying mammalian hosts serving as *T. cruzi* infection sources for the bugs and measuring *T. cruzi* infection prevalence in wild and domestic animals will form a more complete picture of the *T. cruzi* transmission cycle on the island. Connections between human land use and *T. cruzi* transmission must be investigated. Epidemiologic surveys are needed to estimate baseline *T. cruzi* prevalence in humans in Trinidad, and it will be of critical importance to determine whether *T. cruzi* infection contributes to the high rates of heart disease on the island. If after further study, it is confirmed that Chagas disease is indeed a public health concern in Trinidad, a holistic prevention approach that integrates an awareness of

animal hosts, human hosts, and the environment may be necessary in order to effectively address all aspects of this zoonotic NTD.

Supplementary Materials: The following are available online at http://www.mdpi.com/2414-6366/5/4/166/s1, Figure S1: Chagas disease information pamphlet given out during this study. Table S1: Triatomine bug sample site details. File S1: Data for bugs collected, *T. cruzi* diagnosis, and blood meal host sequencing.

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An Integrated Study of *Toxocara* Infection in Honduran Children: Human Seroepidemiology and Environmental Contamination in a Coastal Community

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Abstract: (1) Background: Infections caused by *Toxocara canis* and *T. cati* are considered zoonoses of global importance. Reports from North and South America indicate that human infections are widespread in both continents, but epidemiological information from Central America is still lacking. (2) Methodology: In the present cross-sectional multi-year study, we aimed to undertake the first seroepidemiological and environmental study on toxocariasis in Honduras. This included the determination of seroprevalence of anti-*Toxocara* spp. antibodies in children using a *Toxocara* spp. purified excretory-secretory antigens enzyme-linked immunosorbent assay (TES-ELISA) and a confirmatory Western blot. As well, through statistical analysis including logistic regression we aimed at identifying relevant biological and epidemiological factors associated with seropositivity. The study also entailed detection of parasites' eggs in the soil samples both through Sheather's concentration method and a nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. (3) Results: The study was undertaken in a coastal community of Honduras in 2 different years, 2015 and 2017. A total of 88 healthy schoolchildren completed the study, with participation of 79% (73/92) and 65% (46/71) of the student body in 2015 and 2017, respectively. Thirty-one children participated in both years (i.e., dual participants). Through both serological tests, seropositivity was confirmed in 88.6% (78/88) of children. Due to the high number of seropositives, logistic regression analysis was not possible for most socio-economic and epidemiological variables. Eosinophilia, on the other hand, was associated with seropositivity, independently of other intestinal helminthic infections. Continued seropositivity was observed in most of the dual participants, while seroconversion was determined in 8 of these children. Microscopic examination of soil samples did not yield any positive results. Through nested PCR-RFLP, 3 of the 50 samples (6%) were positive for *Toxocara* spp.; two were identified as *T. canis* and one as *T. cati*. (4) Conclusions: This work documents for the first time, high levels of human exposure to *Toxocara* spp. in Honduras. These findings, along with the country's favorable epidemiological conditions for this zoonosis, emphasize the need for more research to determine whether this infection is underreported in the country.

Keywords: *Toxocara*; toxocariasis; zoonosis; seroepidemiology; neglected tropical diseases; Honduras

1. Introduction

Toxocara spp. are cosmopolitan zoonotic parasites that utilize dogs, cats, foxes and other canids and felids as definitive hosts. When harboring adult worms in their intestine, these animals extensively contaminate their surroundings with their stools containing parasite ova [1]. *Toxocara* species are distributed worldwide, with higher prevalence where infected domestic dogs and cats are allowed to defecate in public spaces [2]. Once fully developed in the environment, *Toxocara* eggs are infectious to definitive hosts as well as to humans. In the latter, however, the parasites do not reach adult stages but rather lodge in tissues as larval stages causing a wide spectrum of pathologies grouped under the clinical term toxocariasis (also called 'toxocarosis') [3].

The significance of human toxocariasis as a disease remains enigmatic, partly due to the multifaceted, nonspecific and cryptic nature of symptoms, making this an insidious disease more closely related to disability and infirmity than mortality. Further, toxocariasis can lead to significant and irreversible damage such as blindness and fibrotic lesions in visceral organs. Recent research suggests that this infection may partially account for cognitive deficits and other neurological complications seen among socioeconomically disadvantaged children [1,4]. There is a strong body of research from Europe and South America and a recent interest resurgence in the United States [5,6].

Conversely, the epidemiological situation of toxocariasis in Central America is largely unknown [7,8]. Even in Latin America and the Caribbean (LAC) nations, where other neglected tropical diseases (NTDs) are well-characterized, toxocariasis has not been consistently studied and no estimates of regional prevalence have been calculated [8]. Despite that data show that *Toxocara* is an important infection in dogs and probably in cats in Central America [9], a recent review by Ma et al. brings to light the paucity of research on human *Toxocara* infection in this particular geographic region [10].

Among Central American countries, Honduras is a country that, due to its climatic and socio-economic characteristics, is endemic for several NTDs and other infections [8]. With over 60% of the population living in poverty (i.e., earning < $2 USD/day) [11–13], and with a large uncontrolled population of domestic cats and dogs, the country offers optimal conditions for *Toxocara* spp. transmission; yet data on toxocariasis is almost non-existent [4,7,8].

In the present study, we aimed to undertake the first seroepidemiological and environmental study on toxocariasis in Honduras. Firstly, we set out to determine the seroprevalence of anti-*Toxocara* spp. antibodies in children as an indicator of exposure to the parasite. Secondly, we sought out to investigate potential associations between seroprevalence and relevant biological and epidemiological factors. Finally, we conducted an environmental sampling to confirm that soil in public spaces could be one source of infection for the study population.

2. Materials and Methods

2.1. Study Design and Population

The present investigation was designed as an exploratory, cross-sectional study. A non-probability, purposive sampling method (based on expert knowledge of the population) was used to obtain the study sample. A minimum sample size was not calculated. Rather, research participants were recruited from a primary school population with high prevalence of soil-transmitted helminth (STH) infections. Two data collection visits took place: in August 2015 and October 2017.

The study was conducted in the village of Santa Cruz del Junco, within the municipality of Tela, department of Atlántida, on the northern Caribbean coast of Honduras.

The municipality has an area of 1196 km^2 and an elevation of 3 m above sea level. The most recent census (2013) by the Honduran National Institute of Statistics recorded a population of 96,758 inhabitants, of which 48.8% were rural residents and 32.8% were ≤14 years old. The same census also documented a poverty index of 51%, with 42% of Tela's inhabitants listing agriculture, animal husbandry or fishing as their main source of income [14]. According to the Köppen Climate Classification, Tela exhibits a tropical rainforest climate, characterized by a lack of dry or wet seasons,

as all months present at least 60 mm of precipitation; a condition that contributes to high levels of humidity. There is also no defined summer or winter in Tela, and it is typically hot and wet year-round [15].

The city of Tela, named after the municipality, is located between 15°47′00″ North latitude and 87°28 00″ West longitude, placing it approximately 67 km north-east of the city of San Pedro Sula, the primary industrial center in Honduras and the nation's second largest city after the capital [14]. The study village is located approximately 11 km from the city's center.

A national survey investigating prevalence and intensities of soil-transmitted helminths (STH) in Honduran schoolchildren had identified Tela as an area with an STH prevalence close to 50% [12]. These conditions are ideal for the transmission of a variety of parasites, including *Toxocara* spp. [16,17].

2.2. Study Sample

Schoolchildren from the village's only public primary school (consisting of grades one through six), were invited to participate in the study, with no exclusion criteria. We focused on school-age children to determine exposure to *Toxocara* spp. based on their known contact with soil and propensity to harbor STH more than adults [8]. It is widely known that *Toxocara* infection is markedly more associated with children, a finding related to certain determinants such as geophagia, playing habits and hygienic practices [18–20].

2.3. Data Collection

Each participant's parent/guardian partook in a face-to-face interview that used a pre-developed questionnaire as a guideline for data collection. The questionnaire was structured into different categories aiming to gather information including demographic and epidemiological data, domestic animal presence in the household, relevant patterns of child behavior and knowledge of parasites.

2.4. Blood Sample Collection and Analysis

Sera collected from each participating child was tested for the detection of anti-*Toxocara* IgG antibodies by way of a commercially available ELISA kit (Diagnostic Automation, Inc./Cortez Diagnostics, Calabasas, CA, USA). According to manufacturer instructions, any sample that yielded an absorbance ≥ 0.3 optical density (OD) units was considered seropositive. All positive ELISA results were confirmed by a Western blot (WB) assay, as well as 3 out of the 8 ELISA-negative samples. As per manufacturer instructions, the presence of two or more bands of low molecular weight were regarded as a positive result (LDBIO Diagnostics, Lyon, France). All assays were conducted according to manufacturer instructions. During the 2015 phase of the study, an eosinophil count was performed by an independent laboratory (Hospital CEMESA clinical laboratory), located in San Pedro Sula city, 1 h-drive from the study site. Eosinophilia was defined as ≥500 eosinophils/μL [21].

2.5. Stool Sample Collection and Analysis

In order to rule out potential serum cross-reactivity due to *Ascaris lumbricoides* infection as well as other parasitic infections characterized by eosinophilia, we analyzed children's stool samples for STH. A single stool sample was collected from each child and analyzed the same day at the local hospital laboratory using the Kato-Katz technique (Vestergaard Frandsen, Lausanne, Switzerland). With a Kato-Katz template delivering 41.7 mg of sample, we used a factor of 24 to obtain the number of eggs per gram (epg) of stool. Kato-Katz smears were microscopically examined after 30 min of clarifying time. Any insufficient or unsuitable (not formed) stool sample was analyzed by direct wet mount examination.

2.6. Soil Sample Collection and Analysis

A total of 50 soil samples, each approximately weighing 30 g, were collected from 5 different sites in the community of Santa Cruz del Junco in 2017 (10 samples per site). Samples were collected from the superficial layer of soil, up to a depth of 5 cm, avoiding any pebbles or grass. Soil samples were exported according to international regulations to Brock University in Canada for further microscopic and molecular analysis (permit number P-2018-00845).

Each sample was dried overnight at 37 °C, sieved with a 150 μm pore size sifter and the resulting refined soil was kept at room temperature in 50 mL conical tubes. Volumes obtained through this process varied from 3 to 13 mL.

The detection of *Toxocara* spp. in the refined samples, was done through a centrifugation/passive flotation technique (i.e., Sheather's) and a nested PCR-RFLP. For the Sheather's technique, samples were analyzed in duplicates as follows: 1 g of soil was transferred to a 15 mL centrifuge tube, mixed with 9 mL of distilled water, vortexed for one minute and then centrifuged at 1500× *g* for 5 min. The resulting supernatant was discarded, and a flotation sucrose solution (Specific Gravity, SG~1.27) was added to the pellet to complete a volume of 10 mL. Tubes were vortexed and filled with the same sucrose solution until a positive meniscus formed. Cover slips were placed on top of the tubes and, after a 60-min period of passive flotation, were examined microscopically at 10× and 40× magnifications for the presence of *Toxocara* spp. eggs.

For the molecular detection of *Toxocara* spp., DNA was extracted from a 250 mg aliquot of the refined soil using a commercial kit (Norgen Soil DNA Isolation Plus Kit cat# 64040, Norgen Biotek Corp., Thorold, ON, Canada). Extraction was performed according to the manufacturer's protocol, with two modifications: (i) a preparatory step consisting of thermal stress, and (ii) a substitution of the beads provided with the kit. The thermal stress procedure entailed three rounds of a freeze-heat cycle, whereby samples were placed at −80 °C for 10 min followed by dry incubation at 90 °C for 10 additional minutes. This thermal stress step was followed by overnight incubation with proteinase K at 56 °C. For the second modification, the plastic beads in each bead tube were replaced with a medley of sterile stainless-steel beads measuring 3 mm, 2 mm and 1.5 mm.

A nested PCR approach, targeting segments of the 18S rRNA gene, was performed using the following primers: PCR 1: NC5f 5′–GTAGGTGAACCTGCGGAAGGATCATT–3′ and NC2r 5′–TTAGTTTCTTTTCCTCCGCT–3′ and PCR 2: FM1f 5′-TTGAGGGGAAATGGGTGAC-3′ and FM2r 5′–TGCTGGAGGCCATATCGT–3′. Each PCR mix contained 10 μM for both forward and reverse primers and 2 μL of template DNA, with a final volume of 25 μL. The cycling conditions for PCR 1 were as follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 6 min. For PCR 2, the cycle involved 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final extension of 72 °C for 5 min. The PCR was optimized for the detection of DNA originating from a single egg using spiked soil samples as well as soil negative controls.

Two restriction enzymes, SalI and MvaI, were used to differentiate between *T. canis* and T. *cati* [13]. For this, amplicons were digested at 37 °C for 3 h. Digestion of the product by SalI 1500 U (ThermoScientific ER0641, ThermoFisher Scientific, Waltham, MA, USA) produced 2 fragments of ~320 bp and ~394 bp in *T. canis* and an undigested band of 736 bp for *T. cati*. While digestion by MvaI (BstNI) 2000 U (ThermoScientific ER0551, ThermoFisher Scientific) yielded 2 fragments of ~600 bp and ~100 bp in *T. cati* and an undigested band of ~700 bp for *T. canis*. Both PCR products and digested fragments were separated in 1.5% agarose gels with ethidium bromide.

2.7. Statistical Analyses

Data were inputted into a Microsoft Excel 2016 spreadsheet (Microsoft Corp., Redmond, WA, USA), cleaned for errors or missing values and exported for analysis to the STATA 13 software package (StataCorp LP., College Station, TX, USA).

For characterization of the study population, we used descriptive statistics for both continuous and categorical variables. Seroprevalence of anti-*Toxocara* antibodies was calculated with the confirmatory test. Assessment of the significance of univariate associations with positive serology (as confirmed by WB) was done by Fisher's exact test. A logistic regression model was applied to investigate the relationship between the various epidemiological risk factors with anti-*Toxocara* antibody presence. Odds ratios (OR) were determined with 95% confidence intervals (CI).

Sensitivity, specificity, positive predictive, and negative predictive values were calculated for the ELISA, compared to the Western blot. The kappa statistic for level of agreement between both diagnostic tools was also evaluated. The level of agreement was interpreted as moderate, strong or almost perfect if the value of kappa was 0.60–0.79, 0.80–0.90 or >0.90, respectively [22]. For the eosinophilia measured in the first phase of the study, a Mann–Whitney *U* test was employed to determine if there was a significant difference in circulating eosinophil counts in seropositive vs. seronegative individuals. Level of significance was established at $\alpha = 0.05$.

2.8. Ethics Statement

Both phases of the present study received ethics clearance from both participating institutions, in Canada (Brock University; file number: 17-032-Sanchez, clearance received 12 September 2017; file number: 14-224-Sanchez, clearance received: 8 May 2015) and Honduras (National Autonomous University of Honduras, Tegucigalpa M.D.C, Honduras. Committee of Ethics, Master's program in Zoonotic and Infectious Diseases School of Microbiology; file number: 04-2017, clearance received: 21 September 2017; file number: 01-2015, clearance received: 1 August 2015). In addition, approval for the implementation of this study was requested from the participating school's principal and grade teachers. Both parental consent and children's assent were also required prior to an individual's participation.

3. Results

3.1. Study Participation and Characterization of the Study Population

A total of 88 different children completed the study. Enrolments were 73 and 46 for 2015 and 2017, respectively, but there were 31 children coincidentally enrolled in both years. These participants were deemed "dual participants". Study participation varied between years: in 2015, participation was 79% of the school enrolment (73/92) whereas for 2017 it was only 65% (46/71). The reason for the latter was a widespread outbreak of hemorrhagic conjunctivitis in Honduras (and throughout Latin America and the Caribbean) due to coxsackievirus A24, which prevented children's attendance to school and their potential enrolment in the study.

The final study sample was comprised of students between the ages of 6–15 years (mean 9.83 years ± 2.25) and 45 (51.1%) girls. A summary of the epidemiological and serological data and children's behavior is displayed in Table 1. An important proportion (71.6%) of interviewed participants reported dog ownership by their family (Table 1). Additionally, most participants with household pets, either dogs or cats, specified that these animals strayed from the household freely (72.6% and 66.7%, respectively). Among the behaviors listed, it is important to mention that 80.4% of children said they had some sort of contact with soil, mostly with soil from the playground adjacent to the school.

3.2. Seroprevalence of Anti-Toxocara Antibodies

Of the 88 serum samples, 80 were ELISA-positive. Western blot results (WB) confirmed that 78 out of the 80 sera were indeed positive for anti-*Toxocara* antibodies and hence, an overall seroprevalence of 88.6% was documented (Table 1). As a manner of internal quality control, we tested with WB 3 out of the 8 (37.5%) ELISA-negative samples. The WB confirmed the absence of specific antibodies in these 3 samples. This was in addition to including the kit's negative controls.

Table 1. Study sample characteristics and laboratory results by year of study. Since there were 31 children who participated in both years, the final sample size amounts to a total of $n = 88$.

Characteristics	2015 $n = 73$ (%)	2017 $n = 46$ (%)	Total $n = 88$ (%) [θ]
Males	35 (48%)	24 (52.2%)	43 (48.9%)
Females	38 (52%)	22 (47.8%)	45 (51.1%)
Age (years), *mean (SD)*	10.3 (1.98)	9.40 (2.25)	9.83 (2.25)
Soil Transmitted Helminthiases (STH) Profile			
Overall STH prevalence	30 (41.1%)	10 (21.7%) [‡‡]	34 (38.6%) [‡‡]
Ascaris lumbricoides infection	10 (13.7%)	2 (4.3%) [‡‡]	10 (11.4%) [‡‡]
Trichuris trichiura infection	26 (35.6%)	10 (21.7%) [‡‡]	30 (34.1%) [‡‡]
Hookworm infection	6 (8.2%)	0 (0%) [‡‡]	6 (6.8%) [‡‡]
Polyparasitic infections	13 (17.8%)	2 (4.3%)	11 (12.5%)
Awareness of STH	57 (78.1%)	24 (52.2%)	62 (70.5%)
Recalled having STH infection	48 (65.7%)	24 (52.2%) [§]	54 (71.1%) [§]
Anti-*Toxocara* Antibody Serology and Eosinophilia			
Positive by TES-ELISA	56 (76.7%)	45 (97.8%)	80 (90.9%)
Confirmed by Western Blot	56 (100%)	43 (95.6%)	78 (97.5%)
Eosinophilia (≥500 eosinophils/μL) *	16 (21.9%)	NA	16 (21.9%) *
Domestic Animal Conditions			
Dog ownership ($n = 88$)	53 (72.6%)	34 (73.9%)	63 (71.6%)
Free-ranging owned dogs	39 (73.5%)	23 (67.6%) [+]	45 (72.6%) [+]
Cat ownership ($n = 46$)	NA	24 (52.1%) [†]	24 (52.1%) [†]
Free-ranging owned cats	NA	16 (66.7%) [†]	16 (66.7%) [†]
Children's Behavior [†]			
Contact with soil in the village	NA	37 (80.4%)	37 (80.4%)
Contact with soil in school playground	NA	41 (91.1%) [““]	41 (91.1%) [““]
Geophagia	NA	2 (4.3%)	2 (4.3%)
Onychophagia	NA	11 (23.9%)	11 (23.9%)
Thumb-sucking	NA	4 (8.7%)	4 (8.7%)
Consume undercooked beef	NA	21 (45.6%)	21 (45.6%)
Consume raw fruits/vegetables	NA	35 (79.5%) [◊◊]	35 (79.5%) [◊◊]

NA: data not collected; * data for 2015 only; [†] data collected for 2017 participants only; [‡‡] two children did not provide satisfactory stool samples for Kato-Katz examination; [§] data not recalled for 10 children; [+] data not recalled for one child; [◊◊] data not recalled for two children; [““] data not recalled for one child; [θ] 31 dual participants counted only once. TES-ELISA: *Toxocara* spp. purified excretory-secretory antigens enzyme-linked immunosorbent assay

Of the seropositive participants, 52.6% (41/78) were males, but neither the univariate analysis nor the logistic regression model identified male sex as statistically significant for seropositivity ($p = 0.09$; OR = 4.43, 95% CI = 0.87–22.42, $p = 0.07$). Due to the high number of children with positive serology (the primary outcome), logistic regression analysis was not possible for most variables. Table 2 shows the results of this analysis for the remaining variables none of which were identified as statistically significant. The results of the dual participants are demonstrated in Table 3. It can be seen that the proportion of seropositive children increased, as 8 of the 31 dual participants seroconverted in the two-year interim (Table 3).

Table 2. Logistic regression analysis of *Toxocara* spp. seropositivity among the studied schoolchildren (n = 88).

Variable	Odds Ratio (OR)	95% Confidence Interval (CI)	p Value
Gender (Male)	4.43	0.87–22.42	0.072
Soil Contact	2.19	0.17–27.96	0.547
Onychophagia	0.61	0.04–7.61	0.698
Raw Beef Consumption	–	–	–
Raw Fruit/Vegetable Consumption	4.25	0.23–78.01	0.330
Dog Ownership	1.80	0.46–7.10	0.396
Dog Age ≤ 1 Year	–	–	–
Playground Contact with Soil	–	–	–

(–): Variable omitted from final model.

Table 3. Serological status of "dual participants": schoolchildren who participated in both 2015 and 2017.

Participants	ELISA Positives 2015 n (%)	Western Blot Confirmed 2015 n (%)	ELISA Positives 2017 n (%)	Western Blot Confirmed 2017 n (%)	Seroconverted by 2017 n (%)
Males (n = 15)	12 (80%)	12 (80%)	15 (100%)	15 (100%)	3 (20%)
Females (n = 16)	9 (56.3%)	9 (56.3%)	15 (93.7%)	14 (87.5%)	5 (31.3%)
Total (n = 31)	21 (67.7%)	21 (67.7%)	30 (96.7%)	29 (93.5%)	8 (26%)

3.3. Toxocara spp. Seropositivity and Eosinophilia

The eosinophil count performed in 2015 revealed eosinophilia (defined as ≥ 500 eosinophils/µL) in 16 children out of the 73 participants (21.9%). A Mann–Whitney U test was done to see if there was a significant difference in the count of circulating eosinophils in schoolchildren who tested seropositive compared to those seronegative (Table 4). To account for possible confounders, the test was also applied when controlling for individuals with any kind of STH infection and *T. trichiura* specifically. A significant difference was found between both subgroups (Table 4). For those that were found free of any STH infection, *Toxocara*-seropositive children averaged 191.3 eosinophils/µL, compared to 101.2 eosinophils/µL in those who were seronegative (p = 0.058). Increased eosinophil levels were also documented for those without trichuriasis (p = 0.035) (Table 4).

Table 4. Mann–Whitney U test results comparing geometric mean of circulating eosinophil levels in seropositive vs. seronegative schoolchildren (n = 73) †.

Geometric Mean (G-Mean) Value	Western Blot Seropositives (95% CI)	Western Blot Seronegatives (95% CI)	p Value
G-Mean * eosinophils/µL	262.1 (211.7–324.4)	101.2 (59.6–171.9)	0.004
G-Mean eosinophils/µL (without STH)	191.3 (141.7–258.2)	101.2 (59.6–171.9)	0.058
G-Mean eosinophils/µL (without *T. trichiura*)	198.5 (151.2–260.5)	101.2 (59.6–171.9)	0.035

† Eosinophilia data only collected in 2015; * G-Mean: geometric mean.

3.4. Comparison of Serodiagnostic Techniques

As an additional step, the performance of the TES-ELISA test used in this study was measured against the Western blot, the current recommended confirmatory test for anti-*Toxocara* antibody detection. The average ELISA sensitivity was 100% (95% CI 95.4–100%) and the average specificity was 80% (95% CI 44.4–97.5%), lower than the 93.7% reported by the manufacturer. With these two parameters, a kappa (κ) statistic was calculated to establish the degree of agreement between the two serodiagnostic tests. In this case, the resulting kappa statistic was κ = 0.87, an indicator of a strong agreement between the two diagnostic tools. Additionally, the average ELISA's positive and negative predictive value (PPV) were 97.5% (95% CI: 91.3–99.7%) and 100% (95% CI: 63.1–100%), respectively.

3.5. Intestinal Parasitic Infections

Two children provided insufficient or unsatisfactory stool samples for the Kato-Katz technique, so the samples were instead analyzed by direct wet mount. Overall, 34 of 88 (38.6%) children were infected with at least one STH species of which *T. trichiura* was the most prevalent (30/88 or 34.1%). Concurrent parasitoses were observed in 11 (12.5%) participants, highlighting the possibility of potential cross-reacting antibodies (Table 1).

3.6. Identification of Toxocara spp. in Soil Samples

Microscopic examination after sucrose concentration of soil samples did not yield any positive results. However, eggs of *T. trichiura* and *A. lumbricoides* were detected with this method in 3 (6%) samples.

In contrast, in the nested PCR-RFLP we identified 3 of the 50 samples (6%) as positive for *Toxocara* spp.; two were identified as *T. canis* and one as *T. cati* DNA. These 3 samples were collected in two of the five collection sites, one of which turned out to be the playground adjacent to the school. These *Toxocara* positive samples were negative for any STH by microscopy after the Sheather's concentration technique.

4. Discussion

Adding to the list of neglected tropical diseases (NTDs) in Honduras, this work documents for the first time, high levels of human exposure to *Toxocara* spp. in the country, and suggests the potential for this infection to be seriously underreported.

To our knowledge, only one clinical case of toxocariasis has been published in Honduras. Puerto-Sanabria et al. [23] described in 2016 a case in a 14-month-old infant with central nervous system involvement. Canine toxocariasis is poorly documented as well, even though the infection is frequently treated at veterinary clinics (Sanchez A, personal observations). Reference to the infection circulating in Honduran puppies is made in a 2002 review paper by Javier and Alger [24]. In addition, through an exhaustive search of Latin American databases and Honduran journals, we were able to find two publications: a study published in the Honduran Medical Journal reporting a toxocariasis prevalence of 3.8% in a sample of 207 dogs (82 owned, 69 from a kennel, and 56 free-roaming) [25], and a conference poster presentation by Valle-Ramirez & collaborators describing *T. canis* infection in 12% of 177 dogs examined [26].

Other than the publications mentioned above, we were not able to find more toxocariasis-related data either published or in the grey literature. Nonetheless, given that canine and human toxocariasis are prevalent in countries with similar climatic and socio-economic characteristics [6], we theorized that this parasitic disease is highly prevalent in Honduras.

The study findings confirmed our hypothesis —at least in the study sample—as we determined an overall seroprevalence of 88.6%. This is a surprisingly high seropositivity, but with the use of a confirmatory test (i.e., Western blot), we are confident that our results are reliable. Such high seroprevalence warrants a clinical investigation as covert toxocariasis would be a serious concern among the studied children [3]. Further, evidence indicates strong links between seropositivity and cognitive and developmental delays [1,4,5,27,28]. To reinforce the plausible link between seropositivity and health effects, we found that after controlling for STH infections, the presence of anti-*Toxocara* antibodies was associated with high levels of eosinophilia. In fact, of 73 children tested in 2015, 16 had high counts of circulating eosinophils and all were seropositive. A difference was observed between the eosinophil geometric means of seropositive children when compared to those of seronegative ones (191.3 eosinophils/µL vs. 101.2 eosinophils/µL, respectively), but this difference did not reach statistical significance ($p = 0.058$).

Many studies have already pointed out eosinophilia as a clinical marker for several helminthic and some protozoal infections [21]. Among helminthic infections, strongyloidiasis is commonly

associated with strong eosinophilic reactions, whereas ascariasis and trichuriasis are known to be eosinophilia-inducing, albeit to a lesser degree [29–31]. *A. lumbricoides*, *T. trichiura*, and hookworm infections showed a pattern of endemic transmission among the studied children, with an overall prevalence of 38.6% across the 2-year period. The higher prevalence of trichuriasis is noteworthy—a fact that we have demonstrated in other Honduran communities and that had prompted us to investigate anthelminthic resistance in light of decades of deworming campaigns in the country [32].

In Honduras, strongyloidiasis is not reported frequently, especially in children [12]. Other parasitoses characterized by eosinophilia such as trichinellosis, filariasis, fascioliasis, echinococcosis, schistosomiasis, etc. [21,29,31–36] are not prevalent in the country.

Eosinophilia, on the other hand, has long been recognized as an important biomarker for toxocariasis [37–40]. In the major clinical presentation, visceral larva migrans (VLM) syndrome, eosinophilia is a distinguishable biomarker that can be drastically elevated [37,41]. In other clinical presentations for instance, ocular larva migrans (OLM), covert toxocariasis or neurotoxocariasis, eosinophilia can still be present, but on average, at lower levels than in VLM [20,37,42]. The concurrent findings of eosinophilia and seropositivity among the studied children are consistent with results from other studies [43–46]. Moreover, the combination of eosinophilia and seropositivity among the studied children may suggest the presence of an active infection (i.e., with viable larvae in tissues) [47]. A clinical and laboratory examination of these children could elucidate whether their seropositive status can be linked to covert (inapparent) or common toxocariasis [3].

Gender of the participating children was not identified as a statistically significant risk factor in our study either. It is interesting to note, however, that the proportion of seropositive boys (41/45, 95.35%) was higher than that of girls (37/45, 82.22%), and that the logistic regression analysis identified boys at about 4-fold increased odds of having anti-*Toxocara* antibodies. It is generally speculated that this association is related to boys' increased exposure to infective eggs in the soil from outdoor activities as well as their proclivity to play and have more contact with animals than girls [48,49].

In terms of epidemiological factors associated to seropositivity, we aimed to identify those postulated in the literature (e.g., soil contact, geophagia, dog ownership, raw beef consumption) but unfortunately, the substantial levels of seropositivity within such a small sample prevented us from detecting statistically significant associations, notably between plausible variables such as soil contact, dog ownership, undercooked beef consumption, or geophagia.

In fact, with almost all children (95%) reporting frequent exposure to soil, and almost 90% of them being seropositive, it would have been surprising to detect any statistical association between these two variables. Still, despite the lack of statistical significance, our logistic regression analysis detected that those reporting soil contact were twice as likely to have anti-*Toxocara* spp. antibodies.

We were also unable to identify the consumption of raw fruits or vegetables as a significant risk factor for anti-*Toxocara* spp. antibody presence. Yet, children who affirmed these dietary habits were found to be at four-times greater odds of being seropositive. A great proportion of children who admitted consuming raw fruits/vegetables were found to be seropositive (34/35, 97.14%).

Lastly, in terms of risk factors, even though the association was not significant, most children reporting family dog ownership (57/63) had almost twice the odds of being seropositive compared to their counterparts. This finding may reflect the considerable impact of these canine populations as a source of contamination, especially for children [50–52]. This is particularly true in areas such as the one studied here, where we observed free-ranging dogs running rampant with unrestricted access to public spaces. It would be important to conduct a canine seroepidemiological survey in the community—and in the country at large—to ascertain the size of the dog population and get an overview of the prevailing health issues including zoonotic pathogens such as toxocariasis.

A unique aspect of the present investigation is the testing of the study population in two different years, which allowed for data collection within the same community at two separate time points (2015 and 2017). Moreover, since the second phase of this study (2017) was carried out within the very same school as two years prior, overlap of some participants was inevitable. Initially, 21 of 31 (67.7%)

dual participants tested seropositive via TES-ELISA in 2015. In 2017, this proportion increased to 30 of the 31 (96.7%). Of these 9 seroconverted participants, the confirmatory Western blot identified one as a false positive, leading to the conclusion that 8 children had been exposed to the parasite during the two-year interim. Seroconversion may be attributed to changes in social and recreational behaviors of children as they age. In the two-year study gap, these children could have experienced dietary changes or new/more frequent contact with definitive or paratenic hosts, contaminated environments or fomites, or acquisition or strengthening of unhygienic habits (e.g., geophagia, inadequate handwashing, etc.) [2,18,53]. Some authors suggest that seroconversion is not intrinsically related to behavior changes. They propose that an increase in antibody titers to detectable levels might be due to the cumulative effect of persistent exposure and infection, or to the constant antigenic stimulation elicited by live larvae in tissues [1]. While in the studied population all these scenarios are possible, the fact remains that there is in the community a continuous presence of infectious sources.

Finally, to further investigate potential sources of infection, we integrated into the study an environmental component. Out of the 50 soil samples collected from 5 community sites, 3 samples (6%) from two sites contained *Toxocara* spp. eggs. Two of the three positive samples were collected from the playground adjacent to the school; an unfenced area that is heavily trafficked by children and animals, even outside school hours. In agreement with worldwide literature [54], our data suggest that the playground could be an important source of exposure to *Toxocara* spp. and if so, installing a fence around its perimeter would help mitigate the risk of exposure.

There are some limitations and strengths to this study. An important limitation that prevents generalization is the small sample size restricted to one cohort of schoolchildren. Not only did the study lack statistical power, but a cluster effect most certainly led to capturing a high prevalence phenomenon. Naturally, a small sample size and the extremely high seropositivity observed hindered meaningful statistical analyses. A second caveat worth mentioning pertains to the lack of clinical data from the studied children. Without investigating the potential health implications of a seropositive status, our study can only suggest that the high levels of exposure to *Toxocara* in this community underscore the need for appropriate attention from the health and veterinary sectors. One more limitation entails not including a canine survey. Although this was considered, we could not secure trained personnel to capture free-ranging dogs to obtain blood and stool samples.

Limitations notwithstanding, the study draws strength from the use of both an ELISA and a confirmatory Western blot, which lends reliability to our seroprevalence data. In addition, we were successful in detecting through PCR *Toxocara* spp. eggs in soil samples of 2 out of 5 collection sites. Further, we were able to identify both *T. canis* and *T. cati* in soil samples, opening research possibilities for the study of feline populations as well. A unique strength of the study is having a sub-sample of participants enrolled in both 2015 and 2017. This allowed us to run a nested comparison at two different time points and identify continued seropositivity as well as seroconversion.

In conclusion, we here present the first serological survey on human toxocariasis in Honduras, filling an important knowledge gap not only in the country but in the Central American region. Next steps should include conducting larger epidemiological, veterinary and clinical investigations using a One Health approach. Such data would inform an initial assessment of the burden of this neglected—almost invisible—zoonotic disease.

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Tropical Medicine and Infectious Disease

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Article

Evaluation of Rapid Extraction Methods Coupled with a Recombinase Polymerase Amplification Assay for Point-of-Need Diagnosis of Post-Kala-Azar Dermal Leishmaniasis

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Abstract: To detect Post-kala-azar leishmaniasis (PKDL) cases, several molecular methods with promising diagnostic efficacy have been developed that involve complicated and expensive DNA extraction methods, thus limiting their application in resource-poor settings. As an alternative, we evaluated two rapid DNA extraction methods and determined their impact on the detection of the parasite DNA using our newly developed recombinase polymerase amplification (RPA) assay. Skin samples were collected from suspected PKDL cases following their diagnosis through national guidelines. The extracted DNA from three skin biopsy samples using three different extraction methods was subjected to RPA and qPCR. The qPCR and RPA assays exhibited highest sensitivities when reference DNA extraction method using Qiagen (Q) kit was followed. In contrast, the sensitivity of the RPA assay dropped to 76.7% and 63.3%, respectively, when the boil & spin (B&S) and SpeedXtract (SE) rapid extraction methods were performed. Despite this compromised sensitivity, the B&S-RPA technique yielded an excellent agreement with both Q-qPCR (k = 0.828) and Q-RPA (k = 0.831) techniques. As expected, the reference DNA extraction method was found to be superior in terms of diagnostic efficacy. Finally, to apply the rapid DNA extraction methods in resource-constrained settings, further methodological refinement is warranted to improve DNA yield and purity through rigorous experiments.

Keywords: post-kala-azar dermal leishmaniasis (PKDL); point-of-need diagnosis; DNA extraction; recombinase polymerase amplification (RPA); real-time PCR

1. Introduction

Post kala-azar dermal leishmaniasis (PKDL) is a sequelae of *Leishmania donovani* infection that mostly affects individuals after successful treatment for visceral leishmaniasis (VL) [1]. PKDL usually manifests as macules (hypopigmented patches), papules, and nodules, or a combination of the three, known as polymorphic skin lesions, mainly on the face, trunk, legs, arms, and genitals [2,3]. For unknown reasons, the incidence of PKDL cases with different types of lesions varies across *L. donovani* endemic regions [2]. In Sudan, 50–60% of treated VL patients develop PKDL within six months, whereas in the Indian subcontinent, PKDL is reported to develop in 5–10% of VL patients within two to four years after treatment [3–5]. Surprisingly, the incidence rate of PKDL increases two fold within five years of completion of VL treatment [6]. In addition, 15–20% of PKDL cases present without a documented history of VL, suggesting that these individuals may have had a prior subclinical *L. donovani* infection that was not detected [4]. PKDL, unlike VL, is not life threatening if it remains untreated, but PKDL patients often unfortunately experience stigma within their society [7,8]. Of further concern, the Leishmania parasites harbored within skin lesions of PKDL patients serve as the known reservoir of VL, and this plays a pivotal role in their interepidemic transmission through sandfly bites, particularly in the Indian subcontinent [9–11]. This vector-borne parasitic disease is anthroponotic in the Indian subcontinent, whereas animal reservoirs are responsible for disease transmission in particular endemic regions [12,13].

Kala-azar elimination programme (KEP) activities in the Indian subcontinent (ISC) have contributed to a remarkable decline in the incidence of kala-azar in recent years, and the KEP is now considered to be in the consolidation phase. However, PKDL is identified as a potential threat to the sustained success of the programme and its ultimate goal of kala azar elimination. Proper diagnosis and management of PKDL has consequently been set as an essential component of the KEP [14,15]. The control programme is facing challenges regarding early diagnosis and treatment of PKDL, however, because of its symptomatic resemblance to other skin diseases such as leprosy, vitiligo, secondary syphilis, and sarcoidosis, and the lack of sensitive field-friendly diagnostic methods [16,17]. The lack of awareness and poor treatment-seeking behavior of PKDL patients further complicate control activities [18,19].

Currently, diagnosis of PKDL relies on clinical assessments with support from parasitological approaches [2]. Direct demonstration of *Leishmania* amastigotes in either slit skin or skin biopsy smear provides 60–100% sensitivity in nodular lesions, but has poor sensitivity in macular lesions (7–50%) [20–22]. Furthermore, several antibody-based serological methods such as direct agglutination test, enzyme linked immunosorbent assay, and rK39-based rapid diagnostic tests (RDT) have been considered as ancillary diagnostic tests for PKDL diagnosis, because all of the treated VL patients give a positive result for antibody-based methods, even after being cured [16,23]. In contrast, molecular methods can detect *L. donovani* DNA, and several conventional as well as real time PCR assays have been developed with high sensitivities and specificities for laboratory diagnosis of both VL and PKDL [20,24]. These methods can help confirm the diagnosis of PKDL in 40–94% of clinically suspected individuals [4,25], and we previously developed a promising real time PCR assay for the diagnosis of PKDL that provided excellent sensitivity (91.2%) for macular PKDL cases in endemic regions of Bangladesh [20]. The application of qPCR in resource-limited settings, including primary and secondary health-care facilities, is challenging because it requires a well-equipped laboratory, trained personnel, and reliable storage conditions for the reagents. Therefore, the need for a user-friendly, design-locked, and field-feasible diagnostic method for PKDL detection remains. In this regard, the recombinase polymerase amplification (RPA) assay has recently emerged as a novel alternative isothermal amplification technology for the detection of nucleic acid [26,27] with the potential to overcome the limitations of poor-resourced settings. RPA provides results faster than conventional and even real time-PCR, despite amplifying nucleic acid at a constant temperature (42 °C), and requiring less expensive and simpler equipment [26]. Due to the many advantages of the RPA assay, we developed an RPA assay for detection of *Leishmania donovani* (LD) parasites that showed absolute sensitivity and

specificity in correspondence with real-time PCR [28]. Furthermore, our newly devised RPA assay can detect both *L. donovani* and *L. infantum*. Eventually, this assay might have broader implications in endemic regions where the disease is both zoonotic and anthroponotic.

In addition to the detection method, an important parameter for the optimum sensitivity of molecular diagnostic approaches is the extraction of high-quality genomic DNA extraction from clinical specimens. Generally, the spin-column-based extraction method produces pure DNA, but requires use of high-speed multiple centrifugation with enhanced washing steps, which is costly and usually not feasible in field settings. On the other hand, the SpeedXtract (SE) method that has been incorporated with the RPA assay in several studies involves a magnetic bead-based lysis protocol to avoid the creation of aerosols and the use of a high-speed centrifuge. The SE method has not, however, been evaluated using skin samples. Alternatively, we found a relatively simple DNA extraction method based on an in-house lysis buffer to be suitable for loop-mediated isothermal assays [29,30]. This rapid boil & spin (B&S) DNA extraction method does not require multiple washing steps with commercial buffers; however, an end-stage centrifugation step is needed to separate DNA containing aqueous layer from the pellet with cellular debris. In an effort to develop a field-friendly diagnostic algorithm for detecting *L. donovani* DNA in skin samples from PKDL patients, we therefore assessed various nucleic acid extraction techniques in combination with an RPA assay.

2. Materials and Methods

2.1. Study Sites and Participants

The study entailed both field and laboratory activities. Field activities were performed at Surja Kanta Kala-azar Research Centre (SKKRC), Mymensingh, Bangladesh, a region highly endemic for VL, and laboratory activities at Emerging Infections and Parasitology, icddr,b, Dhaka, following the approval of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) Institutional Review Board (IRB) (PR-17041). In total, thirty treatment-seeking, suspected PKDL cases residing in the endemic zone were enrolled at the Surya kanta kala-azar research centre (SKKRC), the only specialized hospital for treatment of VL, PKDL, and their associated complications. The majority of the recruited PKDL patient had a history of VL and all exhibited characteristic skin rashes. All PKDL patients were positive in rk39 RDT and were diagnosed based on clinical characteristics by the hospital physician. Following initial examination, each patient was invited to participate in the study, and written informed consent was obtained from either the participant or the legal guardian of children participants before samples were collected. Following standard procedures, the study physician collected three 3 mm skin biopsy samples from each participant. Each biopsy was preserved in NET buffer for subsequent DNA extraction. All PKDL patients were referred for treatment following national guidelines, and each was found to be responsive to treatment. To determine the specificity of our investigative assays, thirty DNA samples were extracted from buffy coat of cured VL patients and were also subjected to laboratory analyses.

2.2. DNA Extraction from Clinical Specimen

DNA was isolated following three DNA extraction methods:

Spin column-based method: This reference method was involved DNA extraction using a QIAamp DNA tissue & blood mini kit (Cat no #69506, Qiagen, Hilden, Germany) according to the manufacturer's protocol with a minor modification: skin biopsy materials were kept at 37 °C overnight after addition of ATL buffer and protease K. The following day, the material was homogenized then incubated at 56 °C for two hours before purification.

SpeedXtract Extraction (SE) method: A simple and rapid blood lysis protocol (SpeedXtract, Qiagen, Hilden, Germany) was modified to suit DNA extraction from skin as follows: 100 µL of Buffer SL and 30 µL of Suspension A (Cat no # 703060, SpeedXtract, Qiagen, Lake Constance, Germany) was added with 3 mm skin punch biopsy in a 2 mL tube and was mixed thoroughly by vortexing for

10 s. Thereafter, the mix was incubated at 95 °C for 10 min and after incubation, the skin biopsy was pressed with grinding pestle and mixed by vortexing. The mix was incubated at 95 °C for another 10 min, then the tube was transferred to a magnetic stand and incubated at room temperature for 1 min. Finally, the supernatant was carefully transferred to a new tube.

Boil & spin (B&S) method: Skin biopsy materials were kept in 37 °C overnight after addition of an in-house-prepared simple lysis buffer (400 mM NaCl, 40 mM Tris pH 6.5, 0.4% SDS) [28] following addition of protease K (Qiagen, Hilden, Germany). The following day, the skin materials were homogenized, then incubated at 70 °C for 15 min. After incubation, the mixture was vortexed, spun, and incubated for 5 min at 95 °C before centrifugation for 3 min at 10,000× *g*. After centrifugation, 30 µL of clear supernatant was transferred to a dilution tube containing 345 µL of PCR-grade water.

2.3. DNA Purity and Concentration

To assess the purity of each extracted DNA sample, OD values at 260 nm and 280 nm were measured by a Thermo Scientific Nanodrop™ 2000 Spectrophotometer (Thermo Scientific, Hilden, Germany) and the ratio calculated (the standard ratio for purified DNA ranges between 1.8 and 2.0). Subsequently, DNA concentration/quantity was determined from the OD value at 260 nm following the standard method [31].

2.4. Molecular Detection of LD-DNA

Recombinase polymerase amplification (RPA) assay: The RPA assay was performed with the extracted DNA samples following the previously published method [28]. In brief, the assay was performed in a 50 µL volume using a TwistAmp exo kit (Product code#TAEXO02KIT, TwistAmp exo kits, TwistDx, Cambridge, UK). Master mix was prepared in a tube with 420 nM of RPA primer, 120 nM of RPA Probe, and 1× rehydration buffer, and was added to the RPA lyophilized pellet. Then, 14 mM Mg acetate was pipetted into the tube lids. Subsequently, template DNA was added to the tubes, and the tube was closed and mixed well. The tubes were immediately placed into the tubescanner (Twista, TwistDx, Cambridge, UK) and incubated for 15 min at 42 °C. The emitted fluorescence signals were measured at 20 s intervals. A combined threshold and first derivative analysis were used for signal interpretation. The total reaction time for RPA was approximately 20 min.

Real time PCR: Real time PCR was performed by a previously published method [32]. Briefly, Taqman primers and probes were designed targeting conserved region of Leishmania REPL repeats (L42486.1) specific for *L. donovani* and *L. infantum* and synthesized by Applied Biosystems [32]. Briefly, a 20 µL reaction mix was prepared, containing 5 µL template, 10 µL of TaqMan® Gene Expression Master Mix (2X), 1 µL preordered primer–probe mix, and PCR grade water. Amplification was performed on a Bio-Rad CFX96 iCycler system with following reaction conditions: 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Samples with cycle threshold (Ct) > 40 were considered negative. The total reaction time for real time PCR was approximately 120 min.

2.5. Reagent Cost and Time Analysis

The reagent costs associated with this study were assessed similarly to previous studies [33–35]. We estimated the cost of each qPCR or RPA reaction including DNA extraction for each individual sample, where only the operational costs of supplies, kit, and reagents were considered. Costs for infrastructure, labor, training, and supervision were not included in the calculation. The time required for each assay was estimated through inclusion of sample processing time prior to each respective DNA extraction method and detection time associated with either qPCR or RPA.

2.6. Statistical Analysis

Parametric and nonparametric tests were performed based on the distribution of data. Kappa and McNemar's test were performed to determine the concordance and discordance among the three extraction methods in combination with the RPA assay. Standard statistical formulas were followed

to determine the sensitivity and specificity of the test with 95% CI. Furthermore, receiver operating characteristic (ROC) curve analysis was performed to determine the accuracy of each of the extraction method when coupled with RPA/qPCR assay. All statistical analyses were performed using SPSS (Version 20.0) and GraphPad Prism (Version 8.1.2). p value < 0.05 was considered as statistically significant.

2.7. Ethics

This study was approved by the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) Institutional Review Board (IRB) and ethical review committee (ERC), research protocol number PR-17041. Informed written consent was collected from each participant or the legal guardian in the case of children.

3. Results

3.1. Participants' Indices

Among the 30 clinically confirmed PKDL cases recruited, 60% were male, and the mean age of the participants was 26.47 ± 12.41 years. Previous history of VL was reported in 93.3% of the PKDL patients, and 23.3% were relapse cases (Table 1). The clinical examination confirmed 22 (73.3%) as macular cases, whereas the remaining cases presented with either nodular (6.7%) or mixed (20%) lesions.

Table 1. Clinical and demographic parameters of the Post-kala-azar leishmaniasis (PKDL) patients enrolled in the study (n = 30).

Variable		Value
Male, *n* (%)		18/30 (60%)
Age in years, mean ± SD		26.47 ± 12.41
Children (<17 years), *n* (%)		9/30 (30%)
Adults (>17 years), *n* (%)		21/30 (70%)
Past history of VL, *n* (%)		28/30 (93.3%)
Past history of PKDL, *n* (%)		7/30 (23.3%)
Type of Rash, *n* (%)	Macular	22/30 (73.3%)
	Nodular	2/30 (6.7%)
	Mixed	6/30 (20%)

3.2. Extraction Method-Based Performance of RPA/qPCR Assay

The qPCR assay detected 26 out of 30 clinically confirmed PKDL patients with a sensitivity of 86.67%, when DNA was extracted with the spin column (Qiagen) method. Likewise, the RPA assay showed elevated performance with a sensitivity of 93.33% when the same DNA extraction method was followed. On the other hand, when the B&S (23/30) and SE (19/30) methods were performed (Figure 1), the RPA assay showed compromised diagnostic efficacy, with a sensitivities of 76.7% and 63.3%, respectively (Table 2). As expected, Q-RPA (20/22) and Q-qPCR (18/22) exhibited a considerably higher positive rate (90.9% and 81.8%) than B&S-RPA and SE-RPA (69.6% and 59.1%) among the macular PKDL cases. In addition, all nodular and mixed cases were detected by both Q-RPA and Q-qPCR, whereas one nodular case was not detected by B&S-RPA, and two mixed cases remained undetected by the SE-RPA assay. Both qPCR and RPA assays showed absolute specificity for all of the extraction methods.

Table 2. Analysis of the performance of three DNA extraction methods with qPCR and recombinase polymerase amplification (RPA) assay.

DNA Extraction Methods	Mean DNA conc. (ng/µL) [95% CI] N = 30	Mean OD 260/280 ratio [95% CI] N = 30	Sensitivity of qPCR [95% CI] (Pos/Neg)	Sensitivity of RPA [95% CI] (Pos/Neg)	Specificity of Q-qPCR and RPA [95% CI] (Pos/Neg)	App. Time (per Sample)		App. Cost (in US$ per Sample)	
						qPCR	RPA	qPCR	RPA
Spin-column method (Qiagen)	21.6 ± 10.4 [17.71–25.49]	1.85 ± 0.09 [1.81–1.88]	86.67% [69.28–96.24%] (26/4)	93.33% [77.93–99.18%] (28/2)	100.00% [88.43–100.00%] (0/30)	17 h [30]	15 h [20] min [26]	16.5 [27]	7.5 [26,33]
Boil & Spin (B&S)	22.9 ± 9.3 [19.47–26.44]	0.91 ± 0.11 [0.87-0.95]	N/A	76.67% [57.72–90.07%] (23/7)		15 h [27,30]	13 h [27]	14.0 [31]	5.0 [31]
SpeedXtract (SE)	140.7 ± 50.5 [121.89–159.63]	0.77 ± 0.22 [0.68–0.85]	N/A	63.33% [43.86–80.07%] (19/11)		2 h 20 min [26]	40 min [26]	15.5 [33]	6.5 [26,33]

The ROC analysis showed the superior diagnostic accuracy of Q-RPA, as presented by the highest value of area under the curve (AUC) (AUC = 0.967). As the least efficacious technique, SE-RPA gave the lowest AUC value (AUC = 0.817) (Figure 2C). Notwithstanding the poor performance, B&S-RPA presented an excellent agreement with both the Q-qPCR (k = 0.828) and Q-RPA (k = 0.831) techniques, whereas SE-RPA showed good agreement with the Q-qPCR (k = 0.755), Q-RPA (k = 0.692), and B&S-RPA (k = 0.635) assay (Table 3) techniques. Furthermore, SE-RPA showed significant discordance with both Q-qPCR (p = 0.02) and Q-RPA (p = 0.004) (Table 3). Overall, among the 30 clinically diagnosed PKDL cases, 16 cases were found to be pan-positive and two cases were pan-negative.

Table 3. Agreement between different DNA extraction methods coupled with the RPA assay and spin-column-based (Qiagen) DNA extraction method together with qPCR assay.

Methods Comparison	Kappa (k)	Agreement	McNemar (p Value)
Q-RPA vs. B&S-RPA	0.831	Excellent	0.06
Q-RPA vs. SE-RPA	0.692	Good	0.004
B&S-RPA vs. SE-RPA	0.635	Good	0.34
Q-RPA vs. Q-qPCR	0.933	Excellent	0.50
B&S-RPA vs. Q-qPCR	0.828	Excellent	0.38
SE-RPA vs. Q-qPCR	0.755	Good	0.02

3.3. Efficiency of DNA Extraction Methods

The DNA concentration was highest for the SpeedXtract method, with an average concentration of 140.7 ± 50.5 ng/µL compared to the Qiagen (21.6 ± 10.4 ng/µL) and boil & spin (22.9 ± 9.3 ng/µL) methods (Table 2). However, the spin-column-based extraction method (Qiagen) gave the most purified DNA with a mean OD 260/280 ratio of 1.85 ± 0.09, while the SE extraction method gave the least purified DNA with a mean OD 260/280 ratio of 0.77 ± 0.22 (Table 2). To be noted, a significant difference was found between RPA positive and negative samples regarding the DNA concentration ($p < 0.001$) and purity ($p < 0.0001$) for the B&S method, which was not observed for the two other methods (Figure 2A,B).

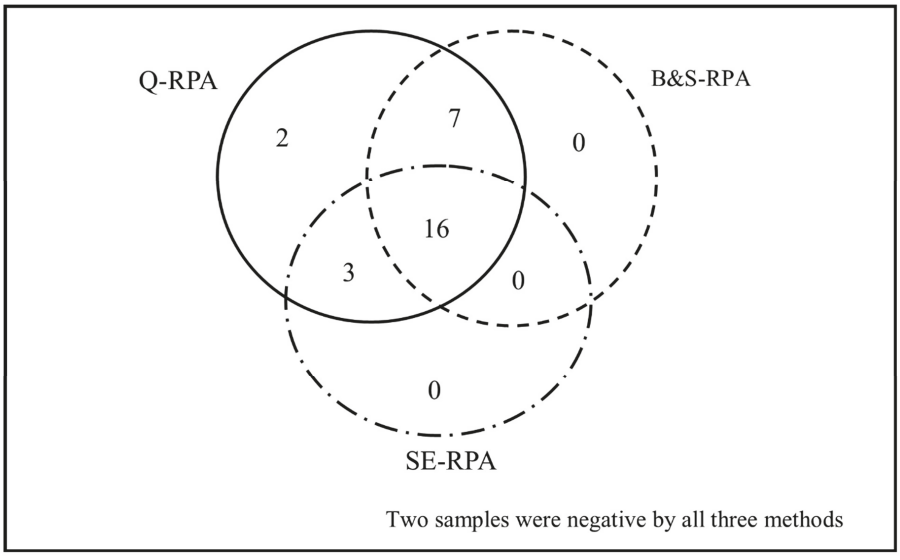
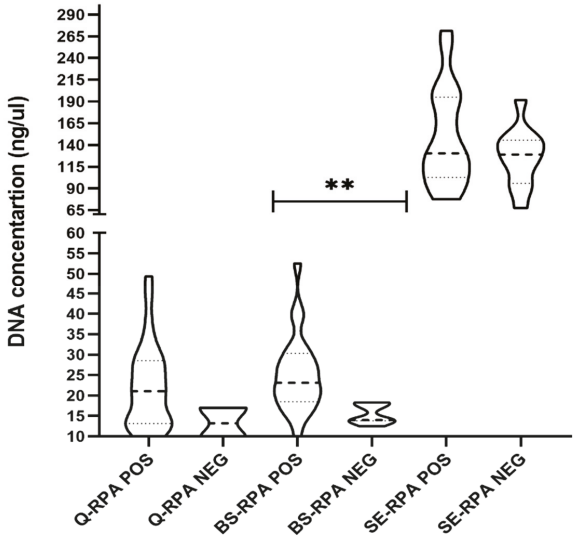
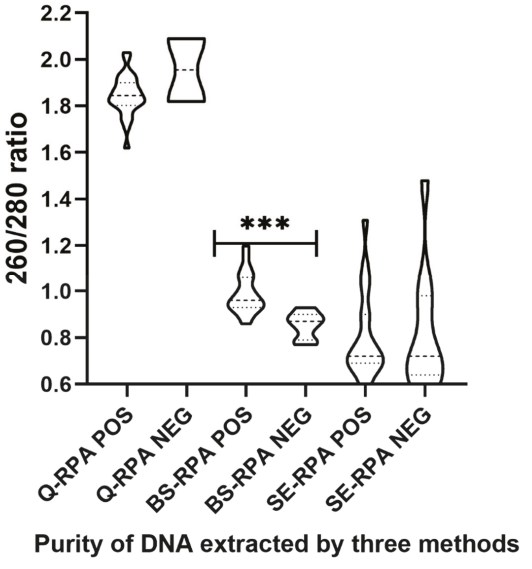


Figure 1. Venn diagram depicting the distribution of Post-kala-azar leishmaniasis detected through Q-RPA, B&S-RPA, and SE-RPA. Among 30 skin biopsy samples from PKDL patients, 28 were positive for *L. donovani* by Q-RPA, whereas 23 and 19 samples were positive by B&S-RPA and SE-RPA, respectively.

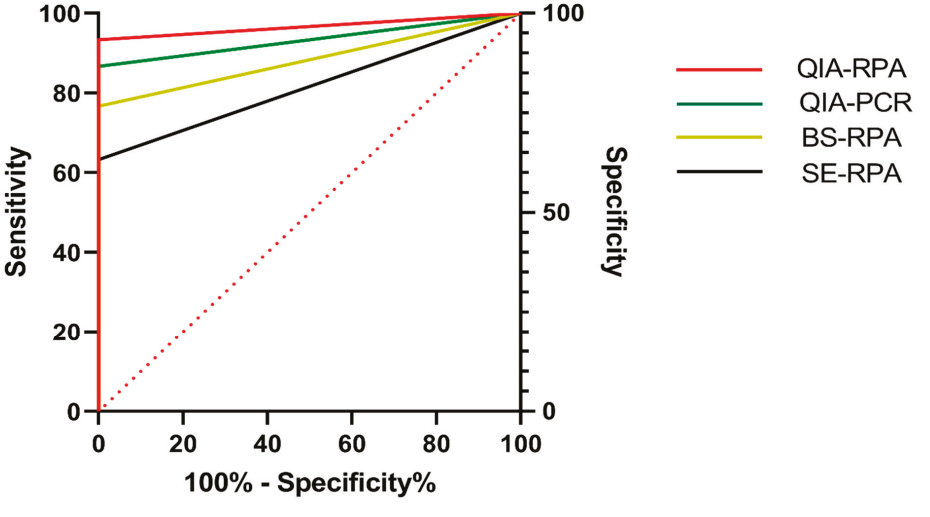


(**A**)

Figure 2. *Cont*.



(**B**)



(**C**)

Figure 2. Illustration of the performance of three DNA extraction methods coupled with the RPA assay. In (**A**), the graph presents the concentration of DNA by the spin column (Qiagen), boil & spin, and SpeedXtract extraction methods, ** $p < 0.001$. In (**B**), the purity of DNA through the spin column (Qiagen), boil & spin, and SpeedXtract extraction methods is shown, *** $p < 0.0001$. In (**C**), the receiver operating characteristic (ROC) curve shows the diagnostic accuracy of the qPCR and RPA assays coupled with the spin-column-based (Qiagen), boil & spin, and SpeedXtract extraction methods for the detection of *Leishmania* parasites in skin biopsy samples.

3.4. Comparison of Assay Time and Cost-Effectiveness

The cost and time estimation analysis showed that the cost and time required for the spin-column-based (Qiagen) DNA extraction method coupled with the qPCR assay were approximately US$16.5 and 17 h, respectively, for each sample, whereas the RPA assay has a lower cost per sample of US$7.5 and requires less performance time (15 h 20 min). We found the B&S-RPA assay to be the least expensive (US$5) assay, and the SE-RPA assay was less time-consuming (40 min) compared to Q-RPA (15 h 20 min) and B&S-RPA (13 h).

4. Discussion

Tremendous declines in the incidence/rate of VL and its mortality due to the initiatives of KEP indicate substantial advancement towards the elimination of kala-azar from the ISC, including Bangladesh. The regional VL initiative is actively pursuing elimination targets by 2020, and Bangladesh has already succeeded in reaching the targets in over 90% of its endemic subdistricts [36–38]. Following the success of the attack phase, the KEP has moved to the consolidation phase with the aim of identifying and controlling potential sources of *L. donovani* infection in endemic areas. Our recent studies have demonstrated that, in addition to VL patients, PKDL cases can competently transmit the parasites to generate new VL cases [10]. PKDL cases are now considered as important reservoirs for parasites and are deemed to be key contributors to interepidemic disease transmission. Therefore, early diagnosis and treatment of PKDL should be prioritized as additional means to control the transmission of *Leishmania* parasites and to ensure elimination is sustained. In a recent review, Zijlstra et al. suggested that validation and implementation of diagnostic methods, including qPCR or isothermal amplification techniques, are essential for diagnosis of PKDL to sustain the success of VL elimination efforts in the ISC [11]. In addition to clinical parameters, qPCR is considered to be the most promising method for diagnosis and assessments of cure [19,23]. The RPA assay has, however, shown comparable diagnostic efficacy to qPCR, while surpassing PCR-based molecular methods with a multitude of practical and technological advantages [28]. A simple and inexpensive method of DNA extraction would make the RPA assay even more feasible and sustainable in poorly resourced settings. We therefore evaluated three DNA extraction methods together with the RPA assay and compared to the reference DNA extraction method (Qiagen) coupled with the qPCR assay to determine if we could generate a field-friendly and cost-effective diagnostic method for diagnosis of PKDL.

Our data indicate that, relative to other extraction methods, considerably higher detection rates are achieved by the RPA (93.3%) and qPCR (86.7%) assays when DNA extraction was performed with the spin-column-based method. We found similar sensitivity (83.4–96.1%) for qPCR in our previous study where only macular cases with low parasite burden were included [19]. In the current study, all nodular and mixed PKDL cases were positive in both RPA and qPCR, a result that is consistent with their higher parasite abundance [15]. These data clearly indicate the promising diagnostic efficacy of Q-RPA assay in detecting Leishmania parasites in skin biopsies from PKDL patients. However, the sensitivity of the RPA assay was compromised when rapid DNA extraction methods such as boil & spin (B&S) and SpeedXtract (SE) were used. Although several studies have reported excellent performance of isothermal amplification-based assays such as LAMP and RPA assays conducted on nucleic acid extracted from whole blood samples by boil & spin and SpeedXtract methods [28–30], our finding is similar to that of an earlier study performed in Sri Lanka on CL patients that reported 65.5% sensitivity of SE-RPA [39].

The comparatively lower sensitivity of boil & spin and SpeedXtract DNA extraction methods in the RPA assay might be attributed to the inherent limitations of these techniques. Notably, the absence of washing steps and deproteination agent, such as proteinase K (SpeedXtract), leads to poor quality DNA with impurities that may inhibit the activity of recombinase and polymerase enzymes in the RPA assay [40]. In addition, the 2.5-fold lower A260/A280 ratio in SE method provides another empirical demonstration of the underperformance of the RPA assay due to the protein impurities in the DNA. Moreover, we noted that skin samples were only partially digested at the end of the

recommended ten minutes in the SE method (notably, the digestion time for two other methods was longer). Surprisingly, we found a significant difference in A260/A280 ratio ($p < 0.0001$) of DNA being extracted by the boil & spin method between RPA positive and negative samples, suggesting promise for this extraction method by increasing the DNA yield. Considering the cost and time for each of the assays, we estimated that the B&S-RPA assay is 3-fold less expensive (at ~US$5 per sample) than Q-qPCR (at ~US$16.5 per sample). On the other hand, SE-RPA assay was far less time-consuming and can produce results significantly faster (within 40 min) than the other combinations. Last but not least, the B&S-RPA assay showed excellent agreement with the Q-RPA and Q-qPCR assays, and the SE-RPA assay presented good agreement with the Q-qPCR, Q-RPA, and B&S-RPA assays (Table 3), further indicating the promise of the B&S-RPA and SE-RPA assays in detecting LD in clinical samples.

The major limitation of this study is that we performed RPA and qPCR assays with DNA extracted by three different DNA extraction methods using different skin biopsies. This might have generated some variance, and an over- or underestimation of the performance of any of the methods, as the parasites are not evenly distributed in the lesions of PKDL patients [5]. The skin biopsy procedure is invasive and requires surgical set-up, which limits the ability to collect multiple biopsies without negatively impacting patient participation and use as an active field-based case detection method. Several recent studies have indicated the satisfactory diagnostic efficacies of the less invasive slit skin, microbiopsy, and fine needle biopsy methods [21,41,42]. Further evidence is required, however, before these invasive sample collection procedures can be applied for diagnosis of PKDL. Another limitation to our study is that, due to ethical issues, we used buffy coat samples from cured VL patients instead of the skin biopsy samples to determine assay specificity. Our previous study provided a basis for the use of blood samples for evaluating the specificity of an index method in PKDL diagnostic studies [19].

Considering the diagnostic performance, operational cost, and feasibility, our data indicate that a spin-column-based (Qiagen) DNA extraction method coupled with the RPA assay can be routinely performed as an alternative to qPCR for diagnosis of PKDL cases. Moreover, this method has the potential to be used for diagnosis of canine leishmaniasis, which implicates the broader application of this rapid molecular technique. In addition, we recommend a refined B&S method as an alternative method to the reference DNA extraction method. Further modification of the SpeedXtract method to achieve better sensitivity could generate a SE-RPA assay that could be used as a point-of-contact tool for rapid diagnosis of PKDL. Further large-scale studies are both warranted and required to generate a Q-RPA assay that can be used for the molecular diagnosis of leishmaniasis involving dermatological complications.

5. Conclusions

The findings of this study demonstrate the superior diagnostic performance of reference DNA extraction method (Qiagen) over the boil & spin (B&S) and SpeedXtract (SE) methods in detecting LD DNA through RPA assay from skin biopsy of PKDL patients. We recommend a spin-column-based (Qiagen) DNA extraction method coupled with the RPA assay as a surrogate mode of diagnosis of PKDL that can be routinely performed as an alternative of qPCR. We believe our findings, and the recommendations we make from them, could help policy-makers adopt a cost-effective diagnostic method for PKDL that could be implemented in resource-limited settings to help the KEP sustain their successes. Further, considering the promises of this assay, clinical studies are imperative for the detection of visceral leishmaniasis both in human and animal reservoirs.

Author Contributions: P.G., D.M., P.N., M.S.D. and A.A.E.W. conceived and designed the study. P.G., R.C., F.H., M.A.A.K., K.F., S.M., D.G., J.B., M.M.-U.-R., M.U.B.R. and R.N. performed the experiments and maintained the data source. PG, RC and M.AA.K. performed the statistical analysis. R.C. and PG drafted the manuscript. P.G., D.M., M.S.D. and A.A.E.W. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no Conflict of Interest.

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Tropical Medicine and Infectious Disease

MDPI

Review

Rabies as a Public Health Concern in India—A Historical Perspective

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Abstract: India bears the highest burden of global dog-mediated human rabies deaths. Despite this, rabies is not notifiable in India and continues to be underprioritised in public health discussions. This review examines the historical treatment of rabies in British India, a disease which has received relatively less attention in the literature on Indian medical history. Human and animal rabies was widespread in British India, and treatment of bite victims imposed a major financial burden on the colonial Government of India. It subsequently became a driver of Pasteurism in India and globally and a key component of British colonial scientific enterprise. Efforts to combat rabies led to the establishment of a wide network of research institutes in India and important breakthroughs in development of rabies vaccines. As a result of these efforts, rabies no longer posed a significant threat to the British, and it declined in administrative and public health priorities in India towards the end of colonial rule—a decline that has yet to be reversed in modern-day India. The review also highlights features of the administrative, scientific and societal approaches to dealing with this disease in British India that persist to this day.

Keywords: stray dogs; Pasteur Institute; vaccination; colonial; British India; Civil Veterinary Department

1. Introduction

"A bite from a mad dog is more dreaded than anything I know; which arises from the horribleness of the disease, the uncertainty of the animal's being mad, or of the infection being received: The not knowing at what period to expect the effects, or to feel confident of having escaped it, keeps the person in a state of cruel suspence (sic) for months, or even years."—Daniel Johnson, Sketches of Field Sports as followed by The Natives of India with observations on the animals (1822).

The World Health Organization (WHO) identifies neglected tropical diseases (NTDs) as a group of communicable diseases affecting over a billion people globally, primarily those living in poverty in low- and middle-income countries in the tropics and subtropics [1], imposing a significant economic burden on these countries. India has the dubious distinction of bearing the largest burden of at least 11 of these NTDs [2]. This list includes rabies, a viral infection caused primarily by the bite of infected hosts belonging to the mammalian order Carnivora (although all mammals, and in exceptional circumstances birds, can be infected), and less frequently the deposition of saliva on wounds or mucous

membranes. In addition, it has the highest mortality rate of all known infectious agents, with nearly all individuals who develop clinical symptoms eventually dying [3]. Rabies has been eliminated (or historically been absent) in Western Europe and several island nations such as Japan, Australia and New Zealand (except for imported cases). In North America, domestic animal rabies in dogs and cats occurs infrequently, mainly through exposure to infected wildlife reservoir hosts [4]. However, in most rabies-endemic countries in Africa and Asia including India, domestic dogs are the main rabies reservoir and source of human exposure [4,5].

Of the 59,000 annual human deaths estimated to occur globally due to dog-mediated rabies, about 35% occur in India [4]. Over three-quarters of cases in India occur in rural communities with poor access to diagnostic facilities and post-exposure prophylaxis, which are key to preventing development of disease [5]. More than 95% of cases are caused by dog bites, largely because of the approximately 60 million stray/free-ranging dogs in the country [6], and many cases of human rabies go undetected, are misdiagnosed or are under-reported [7]. A significant proportion of cases (over one-third in a recent study) are children, and despite the availability of safe and effective vaccines, awareness of and access to post-exposure prophylaxis (PEP), including rabies immunoglobulin, continue to be poor [8].

Despite the high burden of human rabies in India, the disease is not notifiable and a structured surveillance system is yet to be put in place [7]. Rabies is not included in the list of diseases for which surveillance is routinely carried out by states and reported under the Integrated Disease Surveillance Project of the Indian Ministry of Health and Family Welfare [9]. Instead, dog bites and snake bites are to be reported separately (Diseases under surveillance: Presumptive (P form)) [9]. The absence of an organised national or regional system for rabies surveillance compounds the problem of poor availability of human and animal rabies incidence data [10]. Current estimates of the burden of rabies in India (over 20,000 human deaths annually) are based on an epidemiological study conducted in 2003 [5], and even this may be an underestimate of the true disease burden. Another study estimated that 12,700 human deaths from symptomatically identifiable furious rabies occurred in India in 2005 [11]. Most recently, a multicentric survey conducted in 2017 across seven Indian states estimated an annual incidence of animal exposures (bite, scratch or lick) of 1.26%, which was reportedly lower than previous estimates from India [12]. However, the authors acknowledged that, owing to the limited scale of their study, results could not be used to generate a country-level burden of potential rabies exposures.

A perusal of the Five-Year Plans for national development in independent India (post-1947) covering the period 1951 (when the first five-year plan was unveiled) to 2002 reveals that rabies was never prioritised for control. During this period, the very term "rabies" appears only twice—once in the fourth plan (1969–1974) while listing the diseases for which research was conducted at the Central Research Institute at Kasauli and once in the sixth plan (1980–1985) in a brief description of mortality rates of environmentally linked diseases ("Diseases like TB, Gastro-intestinal infections, malaria, filaria, infectious hepatitis, rabbies (sic) and hook worm . . . ") [13]. The 10th plan (2002–2007) mentions the development of a new animal rabies vaccine "being tested for technology transfer", as well as research projects on a number of infectious diseases including rabies, although no further details are provided.

It is only in the 11th plan (2007–2012) that rabies control efforts are first mentioned in the form of pilot projects for the control of human rabies, for which 8.65 crore rupees (~2.1 million US dollars at the time) were allocated. For the first time in a Five-Year Plan, rabies control in animals, animal birth control and vaccination of stray dogs are also mentioned in this plan, as components of animal welfare to be handled by the Animal Welfare Board of India [13].

In 2014, the Ministry of Health and Family Welfare of the Government of India announced funding for a National Rabies Control Programme as part of the 12th five-year plan (2012–2017) [14]. This programme is coordinated by the National Centre for Disease Control, New Delhi, and the Animal Welfare Board of India, with the aim of halving human rabies deaths by the end of 2017. However, little information is available on the achievements of the programme, which finds no mention on the website of the national Ministry of Health and Family Welfare (https://mohfw.gov.in/) or the NITI Aayog (https://niti.gov.in/), which replaced the Planning Commission of India in 2015. A search for the

term "rabies" on the Open Government Data Platform India (https://data.gov.in) returns no results [15]. The annual budget for 2018 presented by the Finance Minister of India allocated 40 crore rupees (approximately 6.13 million US dollars) for a few pilot schemes under the National Rural Health Mission, which includes control of human rabies [16]. This amount has been reduced to 25 crore rupees (approximately 3.51 million US dollars) in 2019 [17] and 2020 [18].

Given this background, it is natural to assume that rabies has always been accorded low priority in India. However, a quick glance through the literature on rabies in pre-independence India (before 1947) suggests otherwise. Rabies was one of several "tropical" afflictions, including cholera, plague, typhoid, tuberculosis, polio and snakebites, that were viewed as serious medical and public health problems, particularly for British residents in India. Consequently, it was subjected to great research and control efforts by the British colonial Government of India (hereafter referred to as GoI), frequently using native Indians as subjects for experimentation to develop and refine vaccines [19]. The effort to combat rabies and other infectious diseases was instrumental in the establishment of a wide network of research institutes in India and some important breakthroughs in development of rabies vaccines. However, the disease appears to have gradually lost priority in scientific circles and the colonial GoI, which may be the basis for its continued neglect in modern India. In this historical context, underlying reasons for the present-day underprioritisation of rabies in post-independence India need to be explored, as these may provide insights into what needs to change in order for rabies control in India to receive the priority and resources it deserves.

We used the search terms "rabies" (and variations of its spelling—"rabbies", "rabeis" and "rabes"), "hydrophobia" and "India" to review a range of historical archives and online and physical documents about rabies in pre-independence India (covering the period from the early 1800s to 1947, when India gained independence from British rule, and the few years immediately after). These included articles published in scientific journals (via PubMed and Google Scholar) and popular magazines, historical documents held at the India Office Records and Private Papers of the British Library and the Wellcome Library at the Wellcome Collection, online archives of the Medical History of British India maintained by the National Library of Scotland (https://digital.nls.uk/indiapapers/), British Parliamentary Papers available via ProQuest UK Parliamentary Papers, historical newspapers available via ProQuest Historical Newspapers and documents available online at the Hathi Trust (www.hathitrust.org) and libraries of the universities of Oxford and Cambridge.

2. Rabies Documentation in Pre-Independence India

As one of the oldest diseases known to man, rabies was widely documented by the earliest human civilisations [20]. A disease akin to rabies was recognised in ancient Indian treatises on health and medicine. The *Susruta Samhita* (*Susruta's Compendium*) is an ancient Indian text of Ayurveda (written between 1000 BCE and the first or second century CE), the Indian system of traditional medicine still practised in most parts of the country. This text details various medical conditions and surgical procedures and discusses in detail the symptoms of rabies in humans bitten by rabid dogs or wildlife, recognising that once symptoms develop in human bite victims, the disease is inevitably fatal [21]. The Mughal emperor Jahangir (1569–1627) is recorded to have noted the symptoms of rabies in an elephant that he owned [22]. It is also highly likely that rabies was documented extensively in the numerous vernacular languages on the Indian subcontinent.

Accounts of British medical and military personnel who worked in India during the 1800s highlight the fact that rabies, also referred to as hydrophobia, was widespread throughout India, responsible for the deaths of numerous Indian, British and European citizens [23–25]. The disease also caused extensive mortality in livestock and pet animals such as purebred dogs owned by British officials [23,26]. These accounts identified the occurrence of large populations of free-roaming ("stray") dogs and to a lesser extent wildlife, predominantly jackals, as the main source of infection [23,25,27]. A collection of observations on life in India by a former surgeon of the East India Company (1822) includes a chapter titled "Observations on hydrophobia and rabid animals" that describes symptoms

in humans in graphic detail [23]. The same chapter and other reports provide detailed descriptions of the progress of rabies in infected pet dogs and wildlife, recounting behavioural changes as symptoms began to manifest [23,26,27]. These symptoms included changes in temperament with increased displays of affection or misdirected aggression, changes in vocalisation which were often noticed by Indian caretakers and changes in appetite, varying from voracious consumption of food to eventual rejection. In one instance, a rabid pup that was bitten by a (presumably rabid) hyena interrupted a dance party, resulting in the party having to be broken up and the pup being killed immediately [23].

These accounts also detail the experiences of British military doctors who often had to treat patients with symptoms of rabies and their agony at having to witness progression of the disease and inevitable death [23]. Much effort was put into discovering ways to treat infected individuals and potential modes of treatment, including traditional Indian cures, were keenly discussed in medical circles [24,28,29]. Even at this time, it was well recognised that treating bite wounds as soon as possible after bites occurred was key to preventing disease progression [23,24]. A letter to the editor *of The Lancet* in 1829 discusses the symptoms of rabies, disputing whether it should also be referred to as "hydrophobia", and possible ways of treatment including bleeding of patients in India [29]. A booklet on Ayurvedic treatments for various illnesses published in 1876 from Cochin, in present-day Kerala, includes symptoms of rabies and traditional treatment methods for exposure to "peppatti visham" (poison from a rabid/mad dog) such as chants, and pills and powders made from plant parts [30]. Various other treatments including Buisson baths [31] and cauterising wounds with caustic agents (e.g., nitric acid) [24,26] have also been documented. Rabies was also a significant health concern for British military personnel stationed in India, and pensions were given to the family of military personnel who died of rabies contracted in the line of duty [32].

Various aspects of rabies also found their way into Indian and British newspapers and magazines, ranging from individual theories about how the disease occurs ("a disease engendered by the practice in England of docking the tails of so many of our sporting and household dogs.") (1861) [33]; reports of incidents of rabid dog bites [34] and outbreaks in wildlife [35]; descriptions of encounters with rabid dogs, symptoms observed and suggested control measures ("lunar caustic . . . apply it well to every wound.") (1859) [36]; and an account of a former army officer who claimed to have successfully recovered from rabies after being exposed in India with a detailed description of his symptoms (1836) [37]. Indian newspapers also reprinted articles about rabies that were initially published in British newspapers [38].

Such news reports and readers' letters to editors make evident that stray dogs, dog bites and rabies were an important public concern, particularly in major cities like Bombay (present-day Mumbai), Poona (present-day Pune), Lahore and Calcutta (present-day Kolkata) where many British and European citizens lived [39–44] and where significant numbers of cases were often reported [45,46]. Public awareness about rabies among British residents would also have been high when rabies was a major threat in Britain during the Victorian era and for a long time after its elimination in 1902 [47]. Complaints about "mad dogs" in India can be found in letters published as early as 1861 [33]. In addition to humans, purebred pet dogs were frequently infected [39,43] and British residents constantly demanded action from authorities to control rabies and stray dog populations [39,48,49], even proposing that private contributions be used to fund control measures [44]. Such concerns about rabies control also need to be located within discourses of sanitation, hygiene and urban improvement that were emerging in British India since the late 1800s [50]. These discourses were a product of the burden imposed by infectious diseases on British army personnel in India [50], and in rapidly expanding cities like Bombay and Calcutta, where epidemics of plague, cholera, measles and smallpox were frequently reported, particularly among the city's poor [51,52].

3. Pasteur Institutes and Rabies Vaccination in British India

The discovery of a rabies vaccine by Louis Pasteur, Emile Roux and other colleagues in 1885 [20,53] was a ground-breaking medical milestone, resulting in the establishment of Pasteur Institutes (PIs) in

various parts of the world for production of rabies vaccines [54]. Initially, individuals exposed to rabies in India had to undertake a long journey to the PI in Paris for treatment, thereby affecting their chances of survival [54]; such journeys were often reported in newspapers [27,34,55–57]. These journeys were a major financial burden for the GoI, by one estimate costing £100 per person treated in 1911 (approximately £12,000 per person in 2019 terms) [58]. Recognising the need to bring rabies treatment to India ("if only for the protection of Europeans, and especially of the troops."), AV Lingard, Imperial bacteriologist at the Imperial Bacteriological Laboratory at Poona proposed in 1891 that "anti-rabic treatment and cure" could be started in the laboratory [54]. There was a public movement in the 1890s in India to gather support for the establishment of such institutes, described in detail by Chakrabarti (2012) [19]. The first PI in India started functioning at the hill station of Kasauli in 1900 under David Semple, a medical officer of the colonial Indian Medical Service [59]. It has been argued that this shift in the choice of locations from hot and humid Pune to the colder environment of Kasauli was driven primarily by a desire to maintain a distance from the native Indian population and to avoid the hot tropical climate of the Indian plains, rather than by scientific considerations [19].

Within a short period, the PI at Kasauli served as the main destination for treating an increasing numbers of individuals, both civilians and soldiers, exposed to rabies using vaccine produced at the institute [60,61] providing significant financial savings to the GoI by avoiding the costs of travel to Paris for treatment [58]. As a result of political pressure to decentralise rabies vaccination [19], PIs (or Pasteur sections within other institutions) were established throughout British India including at Coonoor in South India (1907) [62], Rangoon (in present-day Myanmar) (1915) [63], Shillong (East India) (1917), Bombay (1922) [64], Calcutta (1924) [19] and Patna (1928) [65]. Patients who were exposed through bites would often seek medical advice by sending a telegram to the PI, before deciding on travelling to the institute for treatment [66]. These PIs served thousands of individuals exposed to rabies from all parts of British India and Ceylon (present-day Sri Lanka) [67], even after India gained independence [68,69] and many continue to serve the same function to the present day [70].

Detailed statistics were collected on bite victims presenting for treatment to record information about which species bit them (dog, jackal, etc.), location, number, category and severity of bites (bites on head or face, bites through clothing, etc.), whether they completed the full course of vaccinations and the number of deaths post-vaccination—information which greatly improved scientific understanding about rabies [71]. From 1912, statistics on the number of individuals bitten by rabid animals and not seeking treatment were also compiled at Kasauli [61,72]. Hundreds of animals were also examined every year at PIs, veterinary colleges and other institutions like the Haffkine Institute in Bombay [61,62,73] to confirm a diagnosis of rabies. Thousands of copies of a pamphlet titled "Rabies and antirabic treatment in India" were printed and sent to local governments, with suggestions to translate these into local languages [63]. Updated editions of this pamphlet were published in subsequent years [74,75]. At one point, the Kasauli institute treated more patients every year than any other PI around the world [61,76].

Kasauli also became the site for extensive research into safer and more effective rabies vaccines, since the vaccines in use at the time often resulted in serious neurological complications [77]. One of the key events in the history of rabies vaccines was the development of a phenol-inactivated nerve tissue vaccine by David Semple based on Pasteur's original work and developed through experiments and trials on patients at Kasauli [78]. Used for decades in large parts of the world, production of the Semple vaccine has now been discontinued, although it is still produced for human or animal use in a few countries in Africa [4]. The development and evolution of these and other modern rabies vaccines have been covered in detail elsewhere [19,79,80].

Eventually, post-exposure treatment was also decentralised by opening "outcentres" throughout India, though not without opposition from John Cunningham, the Director of the PI at Kasauli in the 1920s who wanted to expand research on rabies vaccines at the institute [59,81]. A 1923 news report identifies such centres "at Karachi, Allahabad, Ahmednagar, Poona, Belgaum and Karwar" as well as Parel in Bombay [42]. These outcentres made it possible to greatly reduce delays in post-bite treatment, and the mortality rate among treated individuals in 1938 was reported to be 0.52%. By 1938, the Kasauli

institute had over 140 outcentres in the northern provinces and other Indian states, while the Coonoor institute had 223 outcentres in Madras Presidency and southern states [82]. While public funds and government grants initially supported the establishment and functioning of PIs at Kasauli and Coonoor, the effectiveness of and demand for rabies vaccines developed at these centres meant that by the 1920s, these institutions started to function fully as private entities, with most of their income coming from the sale of rabies vaccines to government, municipal and local bodies and state hospitals [82].

4. Rabies Control in Animals

One of the earliest documented pieces of legislation for dealing with stray and rabid dogs in British India is regulation II of 1813, which permitted the destruction of ownerless dogs in Bombay city during specific periods of the year. The strict (and often overenthusiastic) enforcement of this regulation sometimes led to the destruction of owned dogs as well and is closely associated with what has been described as the "Bombay dog riots" of 1832. These riots, which also had communal overtones, have been described in detail elsewhere [83]. Other legislation included 'section 68 of the Cantonment Code of 1912', and provisions in Municipal Acts, which authorised cantonments or municipalities to detain or destroy confirmed or suspected rabid dogs as well as stray dogs. In municipalities, officers of the Civil Veterinary Department (CVD) were authorised to carry out these functions. Some Local Self-Government Acts also permitted issuing rewards for destruction of "noxious animals" [84].

A newspaper report from 1912 describes the system in Madras where dog capture was outsourced to "low caste dog-catchers", and dogs were "painlessly destroyed in a lethal chamber" [85]. It was proposed that a similar system be implemented in Bombay. A news report from 1923 describes the efforts of the Health Department of Bombay municipality in "diminishing the number of dangerous, diseased and stray dogs in the city". This was carried out by a team of "3 sub-inspectors, 2 dog carts, 8 cart drivers, 18 dog catchers and a lethal chamber … in which dogs are destroyed by means of carbonic acid gas". The municipality reportedly spent about 10,000 rupees a year for this purpose, destroying 6579 and 6848 "ownerless dogs" and returning 22 and 6 dogs to their owners in 1921 and 1922, respectively [42]. Similar efforts were also reported from Calcutta [40] and Poona [41]. Lethal chambers and "electrocutors" for destruction of dogs were installed in local bodies—a report of the CVD of Madras Presidency (1929–1930) describes the inspection of lethal chambers in Tiruppur, Coonoor and Pollachi, construction of additional chambers at Erode and Udamalpet and an "electrocutor" at Ootacamund (present-day Ooty) [73]. However, one letter from a reader describing empty dog carts and the number of dogs on the road [39] suggests that such measures may have been no more effective in controlling dog populations and rabies than they were in more contemporary times. These measures and the methods used to kill dogs (carbonic acid gas, strychnine poisoning, clubbing to death, electrocution) [19,86] were opposed by many Indians due to religious reasons [41,83], by Indian vernacular newspapers and many British residents [19]. In addition to dogs, wildlife [74], predominantly jackals [87] were also often destroyed.

A host of other measures targeting owned dogs were largely modelled on measures implemented in Britain in the 1800s that had proven successful in making the country rabies-free by 1902 [47]. Officials recognised that owned dogs in India were often unconfined and thus could be infected with or spread rabies—one report proposed that owned female dogs that were allowed to roam freely when in oestrus resulted in increased dog fights and thus the spread of rabies [88]. Purebred dogs were often allowed to roam freely [89] or used by European soldiers to hunt pariah (unowned mongrels) dogs [19]. Such dogs risked reintroducing rabies into Britain or introducing it into other British colonies when their owners moved around the world [47,90]. Consequently, control measures included enforcing registration of owned dogs (purebred or pariah), levying a "dog tax" [19,86] and issuing badges or discs to be fitted to the collars of owned dogs [89]. Local authorities would thus be able to round up unowned dogs for destruction after 72 h, while straying owned dogs could be returned to their owners. Such a "tax and badge" policy was implemented in Shimla and Mussoorie and reported to function satisfactorily [91], while some local bodies were reportedly not keen on implementing these

measures [92]. In military cantonments, kennels were set aside to isolate suspected rabid dogs for observation and destruction [93]. Some letters to newspapers proposed a ban on importation of dogs into India [49] while others argued that the quarantine of dogs imported into India would be pointless without first controlling rabies in the country [94].

As early as 1899, an annual administration report of the CVD in India highlighted the rise in number of rabies cases presented at Bombay and Lahore veterinary colleges and recommended that the GoI issue orders to prevent its spread in India [95]. However, whether rabies could ever be effectively controlled or eliminated in India appears to have been a contentious topic [96]. At the first meeting of veterinary officers in India, held at Lahore in 1919, veterinarians discussed the challenges of controlling stray dog populations and argued for mandatory licensing of all dogs and systematic destruction of strays [97]. The following resolution was adopted at this meeting—"That it is considered that any suitable measure that can be adopted for reducing and destroying the surplus population of dogs is desirable, but that it does not appear to be possible under the conditions prevailing in India to deal more effectively with the disease. Power should be given to veterinary practitioners to order the detention and destruction of dogs suffering from rabies." Based on this resolution, the GoI appears to have advised local bodies to give veterinary officers relevant powers to perform these functions [84]. At the same time, it voiced doubts about the feasibility of implementing such control measures in rural areas and appears to have left it to municipalities and local self-governments to deal with the problem. This approach appears to have persisted throughout the period of British rule in India and there seem to have been no policies for nationwide rabies control in animals. It was also believed that rabies could not be eliminated in India as it was also maintained in wildlife [91].

After the world's first rabies vaccine for dogs was developed in Japan in 1915, Umeno and Doi developed a single-dose canine rabies vaccine suitable for mass production in 1920 [98,99]. This vaccine was used for mass vaccination of dogs in Japan from 1924–25 [80,99]. In this context, experimental studies to develop a method of veterinary PEP ("anti-rabic inoculation of dogs bitten by rabid dogs"), presumably for valuable owned dogs, had begun at the Punjab Veterinary College in Lahore from 1915, also including horses in later years. These studies were inspired by work being conducted at the PI in Kasauli, fully recognising that the control of rabies in animals would benefit people as well [100]. A report of the Principal of this college in 1922–23 determined that rabies PEP of dogs could be considered an established mode of treatment (while also including caveats about conclusively establishing whether a dog was infected or not) [101]. Similar studies to develop preventive rabies vaccination in dogs were also started at the Madras Veterinary College from 1922 using rabies vaccine from the PI, Coonoor. Initial experiments were reported to be inconclusive because following vaccination, dogs from both experimental and control arms remained healthy after being infected with rabies virus [102].

Even prior to these studies, there are frequent reports of treatment of valuable animals exposed to rabies. For instance, two elephants owned by the Raja of Nilambur that were bitten by a rabid dog were given "anti-rabic treatment" in 1919–20, of which one elephant was confirmed by microscopic examination to have subsequently died of rabies. However the first record of the use of rabies vaccines for veterinary PEP, beyond those reported from the Punjab and Madras Veterinary Colleges, is found in a Madras CVD report from 1923–24, when ten animals were "treated with anti-rabic vaccine" at the veterinary hospital, Calicut (present-day Kozhikode in Kerala) [102].

From 1923, vaccines for veterinary use were issued from the PI, Coonoor, to veterinarians in Madras Presidency and Indian states [82]. The use of PEP to treat valuable animals (owned dogs, livestock at government livestock farms, equines and even a monkey) eventually started throughout most Indian provinces [102,103]. Vaccines were sourced from regional PIs, the Haffkine Institute in Bombay and the HK Mulford drug company in the USA [104]. By the 1930s and 1940s, veterinary PEP was being commonly administered at veterinary colleges and regional veterinary centres [45,73,104,105]. In response to rabies cases in Darjeeling municipality in 1933, legislation was enacted which required dog owners to vaccinate their dogs and to keep them muzzled or on a leash when in public [106].

A letter to a newspaper in 1935 complained that vaccination had failed to prevent the onset of rabies in some owned dogs [43]. Statistics of the number of patients treated at the PIs also reported figures for the number of animals vaccinated [68,107]. Between 1923 and 1948, 14,212 animals had been "prophylactically treated" at the PI in Coonoor [108]—these are all likely to have been owned.

However, the use of veterinary PEP seems to have been restricted to treating valuable owned animals, and mass vaccination of dogs for rabies control does not appear to have been seriously considered in British India. The studies conducted in Japan and the USA on preventive rabies vaccination of dogs as well as vaccination studies conducted at the Madras Veterinary College were discussed at the second meeting of veterinary officers in India, held at Calcutta in 1923. At this meeting, the opinion that rabies could never be eradicated in India persisted, and it was opined that vaccines would be useful only to reduce case numbers [84]. A newspaper report covering the conference stated that "The control of rabies in India constitutes one of the most difficult problems confronting both medical and veterinary authorities. The Conference resolved that the results of investigations upon the prophylactic vaccination of dogs against rabies should be referred to the Central Standing Advisory Committee on Epizootic Diseases and Research with a view to advising Government upon the desirability of enforcing measures of widespread inoculation of dogs against the disease." [109]. We found no records to suggest that mass vaccination of dogs was ever considered by local authorities or the GoI. Mass culling of stray and rabid dogs and registration and, in later years, vaccination of owned dogs appear to have been the most widely implemented rabies control measures in colonial-era India. Such measures were even supported by Mahatma Gandhi, a central figure in the Indian struggle for independence, who stated that "The multiplication of dogs is unnecessary. A roving dog without an owner is a danger to society and a swarm of them is a menace to its very existence" [110].

5. Historic Animal Rabies Incidence in India

Annual administration reports of the CVD provide a detailed picture of the prevalence of animal diseases in British provinces in India. Provinces and presidencies were comprised of districts and municipalities with veterinary dispensaries and diagnostic laboratories, as well as veterinary colleges in some provinces. These institutions reported the number of cases of animal diseases treated or diagnosed. Infectious disease statistics from the earliest CVD reports (1887 onwards) focused solely on those affecting productive livestock (cattle, buffalo, sheep and goats) or equines such as rinderpest, foot-and-mouth disease, haemorrhagic septicaemia, anthrax, surra, strangles, etc. Rabies was mentioned only when it affected these species, and the disease was often included in the category "Other" diseases. It is only from 1903-04 onwards that rabies cases in livestock and dogs, most likely owned, were explicitly recorded in tables of disease summaries. Subsequently, the number of cases recorded increased significantly (Figure 1), which may have been partly driven by a number of provincial administrations framing rules for rabies prevention (e.g., Madras in 1923–1924) [111].

Figure 1 presents the total number of animal rabies cases reported in all species (domestic and wildlife) between 1887–1888 and 1950–1951 across all provinces in British India. Case numbers reported from lower administrative levels (districts and municipalities), from regional veterinary colleges and/or diagnostic laboratories have been combined to present a breakdown by province/state in Figure 2. The Supplementary Information and Table S1 within contain more details on how rabies statistics were presented and compiled [112–115]. These reports indicate that animal rabies was endemic and widespread throughout all provinces in British India, affecting all species of domestic animals, most commonly dogs, and wildlife. Officials frequently highlighted their concerns about alarming increases in rabies cases and recommended implementation of control measures [96,100]. Outbreaks were occasionally reported, necessitating PEP treatment of several animals—for instance, the spike in cases in Bengal province in 1935–1936 when 950 animal rabies cases were reported from all districts [46] (Figure 1). CVD staff were often exposed to rabies and had to undergo PEP at PIs [116].

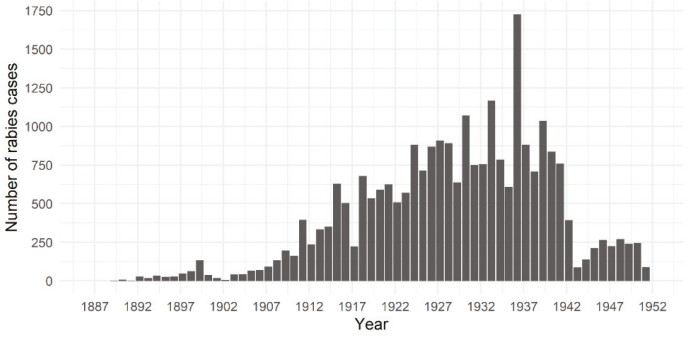
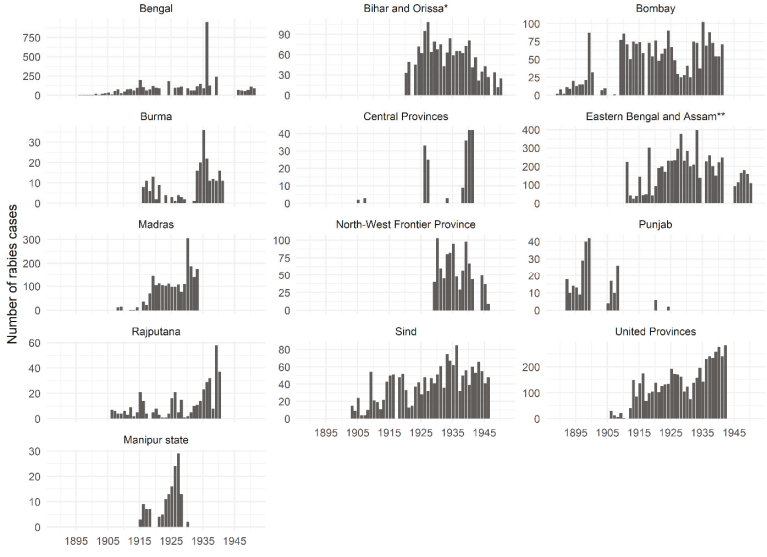


Figure 1. Total number of rabies cases reported each year in all animal species in British India between 1887 and 1951 (annual data span April to the following March, e.g., April 1887 to March 1888). Statistics were compiled from annual reports of the Civil Veterinary Department of the colonial British Government of India, available at https://digital.nls.uk/indiapapers/.



* 1911–12 to 1935–36—Bihar and Orissa provinces, 1936–37 to 1949–50—Bihar only; ** 1907–08 to 1910-11—Eastern Bengal and Assam provinces, 1912–13 to 1949-50—Assam only.

Figure 2. Annual rabies incidence in all animal species between 1887 and 1951 (annual data span April to the following March, e.g., April 1887 to March 1888) in every British province in India (except Baluchistan for which no data were available) and the princely state of Manipur. Statistics were compiled from annual reports of the Civil Veterinary Department of the colonial British Government of India, available at https://digital.nls.uk/indiapapers/. Note the different scale of the *y*-axis for each region.

CVD reports also highlight the wide variation in numbers of rabies cases reported from various provinces (Figure 2). In line with the lack of a consistent policy for rabies control in animals, there was little consistency in reporting of animal rabies cases [115]. Statistics compiled by Chakarabarti (2012) show that between 1880 and 1935 rabies caused an average of 160–170 human deaths per year in Punjab province [19]. However, the few animal rabies cases that are reported from Punjab province appear during the late 1800s and early 1900s, following which cases are reported only sporadically

(Figure 2). It was often acknowledged that reported statistics of animal rabies incidence were likely to be underestimates of the true disease burden [115,117], which was sometimes attributed to a lack of public interest in reporting cases to veterinary officials [92]. There is a marked drop in the number of recorded animal rabies cases after 1941 (Figure 1), possibly because many provinces stopped reporting cases after this period (Figure 2).

Veterinary institutions charged a fee for admission and treatment of cases [118]. Some CVD reports indicate that while there was high demand for rabies PEP, the cost of treatment was unaffordable for poor animal owners and officials were unable to provide it free of cost [45,73]. A 1928–29 CVD report from Madras Presidency describes how poor dog and livestock owners could not afford the cost of PEP for their animals and were advised to administer "indigo blue" instead [114]. In later years, PIs charged for testing brain samples (ten rupees per brain sample in 1933–34), which made this service unaffordable for poor farmers [116]. Such barriers to treatment and diagnosis are likely to have influenced estimates of the true rabies burden and efforts to limit its spread.

6. The Origins of Its Neglect?

Chakrabarti (2012) discusses in detail the various ethical, moral and political debates around scientific research and treatment for rabies in colonial India [19]. It is debatable whether the motives behind the research and development of vaccines and control efforts targeting rabies and other diseases in India were purely altruistic or driven by imperial ambitions and a scientific fascination for tropical illnesses [77,119]. However, it is evident that colonial British governments in India invested time and resources to control rabies (among other diseases) in the country and so the disease could hardly be considered "neglected" from today's perspective.

Given what has been described thus far, what might explain the neglect of rabies by public health practitioners and policymakers in modern-day India? Could it be driven by a perception that rabies had declined sufficiently to justify focusing on other more important human diseases? Dog bites and rabies clearly continued to be major public health concerns in India long after PIs started to save lives from the early 1900s. This is evident from newspaper reports and letters to newspapers from the public [35,39,40,42,93,120,121] as well as official reports and documents of the PIs and the GoI [63,77,122]. Annual reports from the PIs reveal a rapid rise in the number of patients vaccinated against rabies annually. The number of patients treated at the PI in Coonoor and its outcentres increased from under 200 in 1907 to 13,000 in 1935 [77], highlighting the high burden of animal bites in regions served by the institute. This increase was frequently attributed to wider awareness of the availability and benefits of "Pasteurian treatment" rather than any actual increase in rabies [61,63,123].

At the same time, it is not clear that human deaths from rabies reduced substantially in British India. As mentioned above, Chakarabarti (2012) found that around 160 to 170 people died of rabies every year in Punjab province between 1880 and 1935 [19]. This is despite the presence of the PI at Kasauli and the Lahore veterinary college in this province. In 1913, 243 rabies-related deaths were reported from the Central Provinces and Berar [63]. Similarly, 220 deaths were reported in India in 1922, with the report acknowledging that this figure was an underestimate of the true incidence [122]. A letter to the *Times of India* in 1911 speculated that the true number of dog bites and rabies deaths in India was likely to be much higher than those stated in reports from the PI [49]. It was recognised that not everyone completed the full course of post-exposure vaccinations and it was not possible to follow up on outcomes for all patients [122]. As human deaths from rabies in the general population outside those attending the PIs were not systematically recorded, the true death toll may have been much higher.

An examination of human disease statistics and discourses around public health in British India provides some hints about administrative and health priorities vis-à-vis rabies. Notwithstanding concerns raised by the public or officials of the CVD, provincial administrations did not consider rabies to be a concern compared to deaths from other contagious diseases or snakebites [19]. In the late 1800s, mortality statistics from British India included rabies deaths under the broad heading of "Injuries",

which covered a wide variety of conditions (suicide, wounds or accidents, snakebite, injuries caused by wild beasts, etc.) [124,125]. Before the establishment of the PI in India, rabies occasionally killed several soldiers stationed in India (69 deaths in 1879–80, 146 in 1885–86), though considerably fewer than the thousands of deaths every year due to diseases like cholera or smallpox [124,125].

With the advent of PIs and the race within scientific circles around the world to develop safer and more effective rabies vaccines, detailed records began to be maintained in India of the number of people vaccinated from broad ethnic (European and Indian) or religious (Muslim, Hindu, others) groups, those developing complications or dying post-vaccination, the number of patients that completed the full course of vaccination and differences in mortality rates between European and Indian patients [71,126,127]. Such statistics were published in annual reports of PIs [71], scientific journals [91] and, during the early 1900s, regularly included in annual reports presented to the UK Houses of Parliament [61–63,123,127–130]. These efforts served to establish the safety and efficacy of different vaccines being developed at the PIs and improve scientific understanding about rabies. For instance, in a letter written in 1911, W.F. Harvey, the then Director of the PI of Kasauli, recommended the collection of statistics by local bodies on the number of people bitten by rabid animals and who subsequently die without being vaccinated. His aim in suggesting this was to prove that the true mortality rate for rabies was much lower than that reported in statistics from Europe. He stated that this would involve "two or three years' work only", within which period he expected to prove his hypothesis [72]. In several significant respects, India was at the forefront of global research on rabies and the PI at Kasauli was central to this enterprise [19].

Statistical abstracts and reports of burden of illnesses in British India were split into sections—the first dealt with morbidity and mortality in the European Army in India, followed by the Native army (later referred to as the "Indian" army), the general population and jails [61–63,67,126,127,130]. Individuals treated for rabies at PIs were categorised as Europeans (including Eurasians/Anglo-Indians) and natives/Indians and further into soldiers and civilians. The number of Europeans vaccinated against rabies did not increase substantially over the years and few ever died of rabies. The number of Indians vaccinated increased annually, as did the number of recorded deaths (Table 1).

At the same time, overall rabies-related mortality continued to be much lower than mortality from other contagious diseases. Of 22,579 patients vaccinated between 1912 and 1916, only 135 (including 4 Europeans) died. This is in marked contrast with mortality from diseases like cholera (1,259,012 deaths between 1914 and 1917) and plague (1,599,088 deaths in the same period) [131]. At the second meeting of veterinary officers in 1923, it was even remarked that the money spent on rabies control in India would prove more beneficial if diverted for the control of cholera [132]. Indeed, diseases like cholera, plague, smallpox and malaria frequently caused extensive epidemics in India (e.g., the First Cholera Pandemic (1817–1821) [133], the plague epidemic in Bombay (1896) [50]) and high human mortality requiring active interventions by the state [50]. This focus on epidemic diseases would have been in marked contrast with rabies, which was, and continues to be to this day, characterised by fewer cases and only occasional outbreaks in animals [35,46]. Such outbreaks were handled by mass culling of dogs or jackals [87,89] and, following the development of vaccines, by PEP administration to human and animal bite victims.

Table 1. Number of people given rabies post-exposure prophylaxis at various Pasteur Institutes in India. Category totals may not always match as the breakdown of the number of patients treated and the number of deaths was not always explicitly reported.

Year	Numbers Treated (Number of Deaths)			Reference
	European	Native/Indian	Total	
1900–1901 [1]	146 (1)	175 (9)	321 (10)	[130]
1901–1902 [1]	215 (2)	328 (11)	543 (13)	[130]
1902–1903 [1]	269 (1)	315 (12)	584 (12)	[130]
1903–1904 [1]	248 (0)	364 (10)	612 (10)	[130]
1904–1905 [1]	307 (0)	570 (12)	877 (12)	[130]
1905–1906 [1]	342 (2)	803 (19)	1145 (21)	[130]
1906–1907 [1]	452 (2)	846 (17)	1308 (19)	[130]
Interim, 09/08–31/12, 1907 [1]	146 (1)	373 (4)	519 (5)	[130]
1908 [2]	342 (2)	1047 (24)	1729 (26)	[128,130]
1909 [2]	675 (3)	1920 (25)	2595 (28)	[126,130]
1910 [2]	575 (0)	2325 (43)	2900 (43)	[127,130]
1911 [2]	297(1)	2911 (50)	3208 (51)	[130]
1912 [2]	400 (0)	4388 (59)	4788 (59)	[61]
1913 [2]	2 (2)	5271 (66)	5273 (68)	[129]
1914 [2]	NA (1)	NA (60)	5795 (61)	[63]
1915 [2]	468 (1)	6409 (41)	6877 (42)	[123]
1933 [1]	1356 (0)	14,582 (83)	15,938 (83)	[134]
1936 [1]	1357 (0)	NA (97)	NA (97)	[135]
1938 [3]	NA (NA)	NA (NA)	12,396 (21)	[68]

[1] Figures for Kasauli institute only; [2] figures combined for all Pasteur institutes in India, where available; [3] Coonoor institute and its subsidiary centres only; NA—not available.

As mentioned previously, there was also an emerging discourse around sanitation and urban improvement in colonial India from the late 1800s [50–52]. A range of sanitary reforms were implemented from this period, particularly aimed at improving the health of European army personnel who initially suffered significantly higher morbidity and mortality from epidemics in India when compared to Indian soldiers. Sanitary measures such as the provision of piped and filtered water, relocating barracks from swampy areas and improvements in drainage and preventive vaccination against smallpox and plague caused a remarkable and consistent decline in morbidity and mortality among British troops in India [50]. Such sanitary measures would have had little impact on rabies, which would not have been seen to be as amenable to human modification of environmental conditions. Preventive vaccination of humans against rabies, as practised for smallpox, would hardly have been considered necessary, given the sporadic nature of the disease. These epidemiological characteristics of rabies are likely to have greatly influenced colonial perceptions of what diseases could be reasonably controlled through public health interventions.

A similar situation existed with rabies in animals in India. CVD officials were more concerned with the treatment, control and prevention of diseases affecting equines and livestock, which were largely unaffected by rabies. For instance, in 1935-36, when Bengal province experienced rabies outbreaks in multiple districts and recorded 950 cases (Figure 2), the number of livestock deaths from rinderpest and haemorrhagic septicaemia was 35,281 and 3989, respectively [46]. This is unlike the situation reported in Trinidad, for instance, where between 1925 and 1958 repeated outbreaks of rabies transmitted by bats threatened the livestock industry, prompting widespread vaccination campaigns for cattle and efforts to destroy bat populations. As a result, rabies was accorded high government priority for control and elimination in Trinidad with WHO assistance, and much research was conducted on this topic [136]. Dog rabies had also been eliminated in Britain in 1902, and barring occasional outbreaks seeded by dogs brought into the country, rabies ceased to be the significant domestic public health concern it once had been for British politicians and policymakers [47]. This may also have contributed to the gradual loss of interest in investing in rabies control and prevention in British India.

The success of Semple's vaccine in reducing human rabies deaths in India was soon recognised by the global scientific community, and it began to be widely used around the world [81]. By the 1930s, statistics from the PIs continued to be published in scientific journals [108,134,135] but were no longer included in reports to the UK Parliament. During this period, one also finds a return to the practice of including rabies deaths under the head of "Injuries" [137,138]. While research to make the Semple vaccine safer did continue, the key personnel driving this research left the PI or were transferred, and research on other diseases began to take precedence. The PI at Kasauli was shut down in 1939, with work being shifted to the Central Research Institute next door [19]. Research on rabies vaccines and diagnosis continued to be conducted at the Coonoor PI after Indian independence in 1947, spearheaded by the institute's Director N. Veeraraghavan [139], but rabies no longer appears to have been accorded the same priority it once was in British India during the early 20th century.

Thus, despite the importance given to rabies in India with the advent of Pasteurism, a combination of factors is likely to have contributed to its eventual decline in administrative and public health priorities. In particular, the success of Semple's vaccines in preventing human rabies deaths will have influenced administrative officials to prioritise scarce resources towards competing and more pressing public health interventions (e.g., improving sanitation and addressing epidemics). A point to this effect is made in an anecdote in a CVD report about rabies control becoming a priority only "when the deaths amongst humans numbered some scores annually, and a genuine feeling of alarm for personal safety was felt" [140]. In this respect, rabies in British India may have become a victim of its own success, something which is recognised today in Latin America as canine rabies control has become more effective and human mortality has reduced dramatically [141].

7. Impacts on Present-Day Debates in India

Much literature exists on the medical history of a range of infectious diseases that caused major epidemics in British India, including malaria, cholera, plague and smallpox [50,51,142]. This review has examined the historical treatment in British India of rabies as a public health concern, a topic which has received relatively less attention. From being a widespread and untreatable illness, rabies rose to become a driver of Pasteurism in India and globally and a key component of British colonial scientific enterprise. The disease, however, eventually declined in administrative and public health priorities in India towards the end of colonial rule—a decline that has yet to be reversed in modern-day India. In charting this history of rabies, the review highlights features of the colonial administrative, scientific and societal approach to dealing with this disease in India that remarkably persist in the country nearly a century later.

Key among these are the interrelated issues of an absence of a rabies control policy at the national level and of systems for rabies surveillance in humans and animals [7]. Notwithstanding the existence of a National Rabies Control Programme [14], India lacks a well-considered roadmap with realistic milestones to chart progress towards effective national rabies control, let alone elimination by 2030, the target set by the WHO for global elimination of human deaths from dog-mediated rabies [4]. Policy formulation and implementation continue to be the responsibility of states and local bodies, and these are consequently inconsistent. For instance, only two states (Tamil Nadu and Sikkim) have made human rabies notifiable [143]. As was the case in colonial India, animal rabies is not seen as an economically relevant disease affecting animal production systems and hence is not prioritised for control by agriculture or animal husbandry ministries [144].

Rabies in animals was widespread in space and time across British India (Figure 2). The mean number of animal rabies cases recorded between 1903–04 and 1950–51 was 522. This is likely to be an underestimate given that reporting was unsystematic and not mandatory and reported numbers do not include cases from most princely states and territories not under direct British control. The human population of India has risen from 361 million in 1951 to over 1.2 billion in 2011 [145], and the population of dogs has increased correspondingly. In the absence of any comprehensive rabies control measures, it therefore stands to reason that the number of animal rabies cases will also have increased significantly.

Although animal rabies is notifiable in India today, disease reporting is acknowledged to be unreliable even by rabies experts in India [143], and rabies statistics such as those reported by the CVD are difficult to access. This makes the task of estimating the true prevalence in animals extremely difficult. Such gaps in knowledge of the human and animal disease burden and patchy awareness of rabies as a public health threat [146], even among medical health professionals [147], significantly hinder the development of political, scientific and societal urgency to address this burden, particularly in rural areas which bear the biggest brunt [5].

One positive change from the colonial-era approach to rabies control in India is with respect to dog population management (DPM). Although culling of dogs continued to be the mainstay of DPM and rabies control efforts for decades after independence, the Animal Birth Control (ABC) (Dogs) Rules established in 2001 outlawed this inhumane measure [148]. It was replaced by a policy of sterilisation and anti-rabies vaccination (ABC-ARV), carried out once during a dog's lifetime, after which it was returned to its original location. However, in the absence of scientifically robust methods to obtain reliable estimates of dog population sizes, ABC-ARV is implemented in a haphazard and uncoordinated manner across local bodies and states, involving various public [149,150] and private entities [151]. The policy also does not account for the need to revaccinate sterilised dogs to maintain anti-rabies immunity in the dog population and, consequently, the possibility that these dogs may continue to bite and transmit rabies to other dogs and people [152]. Rule 10 of the ABC Rules prohibits the euthanasia of dogs suspected to be rabid, instead requiring such dogs to be isolated until they die naturally of rabies [148], followed by laboratory confirmation of disease [153]. This is clearly a welfare issue for infected dogs. Enforcement of the ABC rules is also inconsistent, and culling of dogs still occurs occasionally throughout India [154], often in retaliation to incidents of injuries or deaths from dog attacks [155].

There is also a flawed perception of ABC-ARV as more than just a DPM tool. Consequently, this measure is increasingly viewed as the primary rabies control measure in India, perceived to be unsuccessful in reducing disease only because of ineffective and/or inadequate implementation [156]. This perception is despite the fact that the WHO itself recommends sterilisation of dogs only as a supportive measure to maintain levels of rabies vaccination coverage achieved through mass rabies vaccination, accepted as the most scientific method for rabies control [4]. A recent study was unable to evaluate the role of surgical sterilisation in controlling dog rabies due to poor data collection or reporting and recommended that mass vaccination should continue to be the control method of choice [157]. The ABC-ARV policy also finds support through discourses that argue for the continued existence of street dogs as "integral inhabitants of the multispecies city" [156].

Wang (2019) describes the conflict that existed in New York City from the early 20th century, between the American Society for Prevention of Cruelty to Animals and the Department of Health, over population control and muzzling of the city's free-roaming dogs [158]. No such conflict appears to have existed over dog culling in colonial India. On the contrary, there is in India today an impasse, along the lines of that which existed in New York, between two conflicting perspectives of the place of dogs on the streets, with direct impacts on rabies control efforts. On the one hand is the view that there should be a holistic approach to control stray dog populations on public health, wildlife conservation [159] and animal welfare grounds. This approach would require enforcing responsible dog ownership, civic waste and humane dog population management and a national mass rabies vaccination programme [4,152], eventually leading to the elimination of free-roaming dogs. On the other hand is the view, held primarily by animal welfare campaigners, that dogs have the right to exist on the streets and to be fed by people [160]. This latter view consequently favours ABC-ARV as the most appropriate DPM and rabies control measure, notwithstanding its drawbacks. Nadal (2020) discusses various aspects of the conflicting perspectives about DPM and rabies control, as well as the complex social, cultural and political contexts within which people and dogs interact in two major cities in North India [161].

In this respect, the ABC-ARV policy has made it difficult to adopt comprehensive measures to deal with the persistent threat of rabies posed by the large populations of unowned free-roaming dogs in

India [144], particularly implementation of mass dog vaccination. Despite evidence from the 1920s that mass vaccination of dogs successfully reduces rabies incidence and can eliminate it [98,99], there were no attempts to implement such a measure in colonial India. Instead, it was widely considered that rabies could never be effectively eliminated in India. This perception continues to hold sway at the highest levels of government to this day with the view that logistical constraints make mass vaccination of dogs unfeasible in India [162]. It is left to state administrations to implement mass vaccination policies, commonly in partnership with nongovernmental organisations [163,164].

In another unfortunate parallel with the colonial era, there is little emphasis on promoting responsible dog ownership practices such as confinement and vaccination of owned dogs in India. It is unclear how successful attempts were by colonial administrations to enforce registration and identification of owned dogs. While several local bodies have now made such measures mandatory in India, they are poorly enforced [165]. Rabies vaccination, while largely confined to owned dogs, primarily valuable pure breeds, is also not mandatory. This often results in poor compliance with vaccination regimens [165], especially in the case of owned mongrel dogs (i.e., those that do not belong to any specific breed), from poorer households who have greater difficulty accessing veterinary services. A high proportion of this latter category of dogs are also poorly confined, frequently resulting in the birth of unwanted pups and increased risk of contracting rabies from interactions with free-roaming unowned dogs [166].

8. Conclusions

Notwithstanding poor availability of disease data, the case may be made that rabies does not impose the kind of human health burden in India that diseases such as tuberculosis, malaria and HIV do. Consequently, rabies control may not be seen as a cost-effective public health investment, a view that was certainly shared by public health practitioners in British India [132]. Such a perspective, however, fails to consider the impact of rabies on individuals from rural backgrounds, particularly children [8], and the near certainty of death in the absence of access to treatment before symptoms appear. As an entirely vaccine-preventable disease disproportionately affecting the poor in low- and middle-income countries, preventing unnecessary human rabies deaths and suffering by addressing barriers to access to human PEP is an important means of achieving social justice [167]. At the same time, the cost of human PEP provision can be substantial (30 million US dollars over an unspecified timeframe in India, by one estimate) [143]. Rabies control through mass dog vaccination has been consistently shown to be more cost-effective in preventing human rabies deaths [168,169]. With the science and tools for rabies control already existing, rabies elimination is low-hanging fruit and a textbook example of the One Health approach in action. This is well recognised even in India, with zoonotic disease prioritisation exercises frequently identifying rabies as one of the main diseases for targeting control efforts [170,171]. Political will has been key to implementing effective control measures in many countries around the world [4] and is the primary factor currently hindering progress on this front in India today.

Rabies in British India was clearly not a "neglected" public health concern. Early rabies vaccines were highly effective in saving human lives, although there remained a poor understanding of the true disease burden in Indian society. These factors, combined with changing priorities of colonial British governments, in all likelihood contributed to a progressive loss of priority of rabies control in the face of the vast array of competing infectious disease and public health challenges in British India. It may be possible to argue that the current neglect of rabies in India is a legacy, albeit unintended, of British colonial rule, but this clearly is no justification for carrying on in the same vein. Current public health professionals and policy makers should look to the extensive historic and current scientific literature on evidence-based rabies control measures to formulate a strategy to achieve the lofty goal of elimination of dog-mediated human rabies deaths by 2030. Equally crucial will be measures to deal with the extensive free-roaming dog population in India, without which rabies control efforts will become unsustainable in the long run.

Supplementary Materials: The following are available online at http://www.mdpi.com/2414-6366/5/4/162/s1, Table S1. Annual rabies incidence in all animal species between 1887–88 and 1950–51 (denoted 1888 and 1951, respectively) in all British provinces in India and the princely state of Manipur. Statistics were compiled from annual reports of the Civil Veterinary Department of the colonial British Government of India, available at https://digital.nls.uk/indiapapers/.

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Tropical Medicine and Infectious Disease

MDPI

Review

A One Health Approach for Guinea Worm Disease Control: Scope and Opportunities

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Abstract: Guinea worm disease (GWD) is a neglected tropical disease that was targeted for eradication several decades ago because of its limited geographical distribution, predictable seasonality, straightforward diagnosis, and exclusive infection of humans. However, a growing body of evidence challenges this last attribute and suggests that GWD can affect both humans and animal populations. The One Health approach emphasizes the relatedness of human, animal, and environmental health. We reviewed epidemiological evidence that could support the utility of a One Health approach for GWD control in the six countries that have reported human GWD cases since 2015—Angola, Cameroon, Chad, Ethiopia, Mali, and South Sudan. Human GWD cases have dramatically declined, but recent years have seen a gradual increase in human case counts, cases in new geographies, and a rapidly growing number of animal infections. Taken together, these suggest a need for an adjusted approach for eradicating GWD using a framework rooted in One Health, dedicated to improving disease surveillance and in animals; pinpointing the dominant routes of infection in animals; elucidating the disease burden in animals; determining transmission risk factors among animals and from animals to humans; and identifying practical ways to foster horizontal and multidisciplinary approaches.

Keywords: animal health; *Dracunculus medinensis*; guinea worm; human health; infectious disease; one health; zoonoses

1. Introduction

Guinea worm disease (GWD), also known as dracunculiasis or Medina worm disease, is a neglected tropical disease (NTD) caused by a nematode, *Dracunculus medinensis*. Humans usually become infected by ingesting water containing copepods (small aquatic crustaceans) infected with Dracunculus larvae [1]. While the copepods are killed in the stomach, the larvae mature into adult Guinea worms and copulate. Male worms die following copulation, but approximately one-year post-infection, adult female Guinea worms migrate to the skin and breach the surface through a blister. When these wounds are exposed to a water source, the female worm releases embryos (i.e., L1 larvae) and the cycle repeats.

Secondary infections are common, and complications can result in abscess formation, tetanus, septic arthritis, or systemic sepsis [1]. Though rarely fatal, GWD can cause permanent disability and impose major economic burdens [1,2]. Like many other NTDs, GWD primarily affects poor, rural communities. Neither a vaccine nor effective medical prophylaxis is currently available [3,4]. Case management is limited to carefully and safely removing the whole worm and tending the exit wound to prevent infection.

Following the success of the smallpox eradication campaign in 1979, public health experts identified GWD as a candidate for eradication [4]. GWD was considered a suitable candidate because its geographical distribution was limited to tropical or subtropical areas, it adhered to seasonal

transmission patterns [5,6], the diagnosis was straightforward [7], and, critically, there were no known animal reservoirs [3,4].

Led by the Carter Center and supported by the World Health Organization, United Nations Children's Fund, and United States Centers for Disease Control and Prevention (CDC), the Global Dracunculiasis Eradication Campaign began in 1980 and has significantly reduced the global burden of the disease [8]. Since it began, when there were more than 3 million cases of GWD in 20 countries, the eradication campaign has dramatically reduced GWD prevalence and as few as 22 cases were reported in four countries in 2015 [9]. However, recent years have seen an increase in the number of reported cases, as well as human cases and animal infections reported in new countries and countries that had previously been certified free of GWD. Consequently, the eradication target has been delayed until 2030 [10].

These delays are the result of numerous factors, including conflict and social unrest, that have limited the ability of field teams to conduct active searches for cases [2,10–12]. However, an emerging body of evidence also suggests that eradication efforts have been further impeded by the presence of other non-human animal species (hereafter "animals") that act as hosts and maintain transmission. Dogs, cats, and baboons, and potentially fish and frogs, are now recognized as playing a role in the parasite's transmission to human populations [3,13–19]. Of these, widespread reservoir infections in dogs are considered the largest threat to eradication efforts [11,20]. Adult Guinea worms emerging from humans and these other animals are genetically indistinguishable, with evidence that worms emerging from animals have subsequently caused infections in humans by releasing larvae into shared water sources [13].

That multiple countries now report animal infections underscores the need for novel intervention and eradication strategies [21]. The identification of the worm in so many species complicates eradication and necessitates an interdisciplinary approach. One Health is a concept that acknowledges the relatedness of human, animal, and environmental health and the importance of interdisciplinary efforts in achieving positive health outcomes across these populations [22,23]. Although the body of work to eradicate GWD has been substantial, relatively little research has been undertaken to elucidate the role that animals may play in transmission or to isolate the impact of specific interventions on the number of animal infections. Accordingly, this paper reviews the epidemiological evidence as it relates to animal epidemiology, and in turn to potential animal-level interventions toward the goal of human eradiation, and creates a conceptual framework for how such an approach could be applied to create new GWD control paradigms that support eradication efforts.

2. Materials and Methods

We conducted a review to identify GWD surveillance data for humans and animals and to identify interventions being implemented to control the spread of disease. We performed a literature review using the PubMed and Web of Science databases. Synonyms for "Dracunculiasis", "dog", "cat", "baboon", "frog", and "fish" were combined with a list of the countries that have reported GWD cases since 2015 (i.e., Angola, Cameroon, Chad, Ethiopia, Mali, and South Sudan). A supplementary file provides the complete search syntax (Supplementary Table S1). Our last search was conducted in January 2020.

We attempted to obtain detailed monthly human and animal surveillance data, including through outreach to the Carter Center, but found no sources. To address this, we collected all Guinea Worm Wrap-Up reports—reports periodically co-published by the Carter Center and the U.S. CDC that contain human and animal surveillance data—published between January 2015 and January 2020 and reviewed their surveillance data for human cases and animal infections [24]. The monthly human surveillance data were robust, but the monthly animal surveillance data were not publicly reported. As a result, for animals, we gathered monthly surveillance data when available, but focused efforts primarily on annual data. These were supplemented with data from the World Health Organization. Gathered data were compiled in a Microsoft Excel spreadsheet (Supplementary Table S2).

We then reviewed and analyzed the identified peer-reviewed literature and surveillance data to synthesize a conceptual framework for how such an approach that emphasizes the nexus of human, animal, and environmental health could enhance efforts to eradicate GWD.

3. Results

3.1. Epidemiological Data

In 2010, a collective 1786 human GWD cases were reported in the five countries considered in this study, with a majority of these coming from South Sudan (Table 1). By 2015, total reported human cases declined to 22, from which point they gradually rose to reach 53 in 2019 (Figure 1), with a majority of these cases coming from Chad. Four countries reported human GWD cases from 2010–2015, three from 2016–2018, and four in 2019. Angola and Chad were the only two countries to report more human GWD cases in 2019 than in 2010. After no previous reports of cases (with the caveat that no GWD-specific case search programs had ever been active in the country), Angola reported one human GWD case in 2018 and another in 2019 (Table 1). Chad reported 10 human cases in 2010, and a low of 9 cases in 2015, before reported cases began to increase, reaching 47 in 2019.

A variety of mammalian species have long been established as potential reservoirs of GWD; however, there was limited evidence that these species were transmitting Guinea worm to humans [3,15]. Sporadic infections in dogs and other animals have been historically reported in Cote d'Ivoire, Ghana, India, Kazakhstan, and Pakistan, but studies to definitively assess animals as a transmission risk factor for humans are limited. In the countries listed, infections in animals are thought to have ceased after human infections were eliminated [28]. Whether these animal infections were instances of anthroponosis (i.e., the transmission of a pathogen from humans to a nonhuman species) that were then interrupted via interventions in the human host is not known as studies have not assessed this question.

Table 1. Reported Human Cases of Guinea Worm Disease in Considered Countries, 2010–2019.

Year	Country						Total
	Angola	Cameroon	Chad	Ethiopia	Mali	South Sudan	
2010	0	0	10	21	57	1698	1786
2011	0	0	10	8	12	1028	1058
2012	0	0	10	4	4	521	539 [1]
2013	0	0	14	7	11	113	145 [2]
2014	0	0	13	3	40	70	126
2015	0	0	9	3	5	5	22
2016	0	0	16	3	0	6	25
2017	0	0	15	15	0	0	30
2018	1	0	17	0	0	10	28
2019	1	1 [3]	47	0	0	4	53

[1] An additional three cases were reported in Niger in 2012, but none have been reported since. These were thought to have been imported from Mali [25]. [2] An additional three cases were reported in Sudan in 2013, but none have been reported since. While the cases were not classified as imported, they were detected in an area of southwest Sudan that borders South Sudan and the Central African Republic [26]. [3] A 2019 case in Cameroon was found close to the Chad border; the origin of infection remains unclear, though human cases and reports of animal infections from the same part of Cameroon could suggest localized transmission [27].

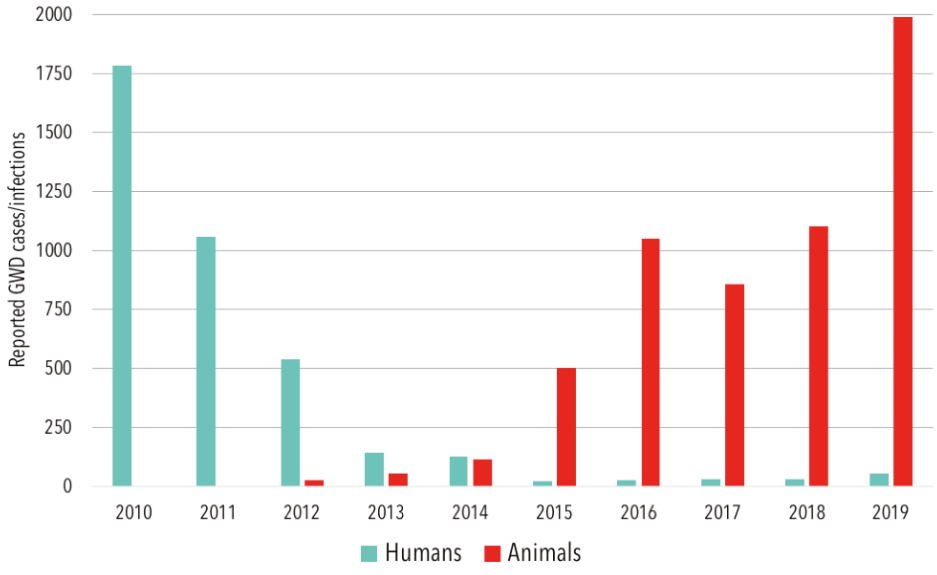

Figure 1. The total annual reported human cases and animal infections of Guinea worm disease in Angola, Cameroon, Chad, Ethiopia, Mali, and South Sudan, 2010–2019.

There has been an increase in reported animal Guinea worm infections since 2015. Most reported animal Guinea worm infections have involved dogs in Chad. The total number of reported canine infections has grown from 498 in 2015 to 1943 infections in 2019 (Table 2). The pathways by which dogs are infected remain unconfirmed, but it is hypothesized they can acquire Guinea worm infection in the same way as humans (i.e., from drinking water containing infected copepods), or possibly by consuming infected paratenic hosts [15]. The consumption of raw fish entrails, discarded by humans during food preparation, has been considered a potential source of transmission and addressed by the human behavioral intervention of promoting burial of entrails in all known-endemic villages since 2015 [21]. A study conducted in Chad found that dogs in households where water was provided for animals were less likely to have had Guinea worm and that dogs with a higher proportion of fish in their diets were more likely to have had Guinea worm [19]. However, dogs with less fish in their diets and those more likely to consume provided water may also live further from natural water sources and therefore less likely to be infected via the traditional waterborne route. A similar study conducted in Ethiopia found no evidence to support the hypothesized paratenic host transmission pathway [29]. Further, although the phenomenon of canine infections with Guinea worm was only recently realized, evidence suggests that this was the result of insufficient surveillance rather than the absence of infection [13].

Guinea worm infections have also been reported in cats, baboons, a leopard [27], and a donkey [28]. The incident involving the leopard was later removed from official counts because the worm was found while dissecting the dead animal, and had not emerged as is required for official case counts [27,30]. Excluding dogs, animal infection counts rose from 5 reported infections in 2015 to 53 in 2019 (Table 2).

Chad has reported the most animal infections, including dogs, of any country and there appears to be a temporal alignment between patterns of reported human cases and animal infections (Figure 2). After first reporting 27 infections in dogs in 2012 [32], the number has risen steadily, peaking at 1927 infections in 2019 [30]. Humans and dogs are infected by shared populations of worms in Chad and it has been hypothesized that this may be driving the increase in human GWD cases [13,15]. Chad also reported four Guinea worm infections in cats in 2015, rising to 46 infections in 2019 following intensified education and surveillance efforts targeting cats.

Table 2. Reported Animal Infections of Guinea Worm Disease in Considered Countries, 2015–2019; (Canine Infections).

Year	Country						Total
	Angola	Cameroon	Chad	Ethiopia	Mali	South Sudan	
2015	0	0	487 (483)	14 (13)	1 (1)	1 (1)	503 (498)
2016	0	0	1022 (1011)	16 (14)	11 (11)	0	1049 (1036)
2017	0	0	830 (817)	15 (11)	10 (9)	0	855 (837)
2018	0	1 (1)	1065 (1040)	17 (11)	20 (18)	0	1103 (1070)
2019	1 (1)	5 (5) [1]	1973 (1927)	8 (2)	9 (8)	0	1996 (1943)

[1] This number includes only reported infections explicitly mentioned in a Carter Center update [31], and does not include other unconfirmed rumors discovered by WHO investigators.

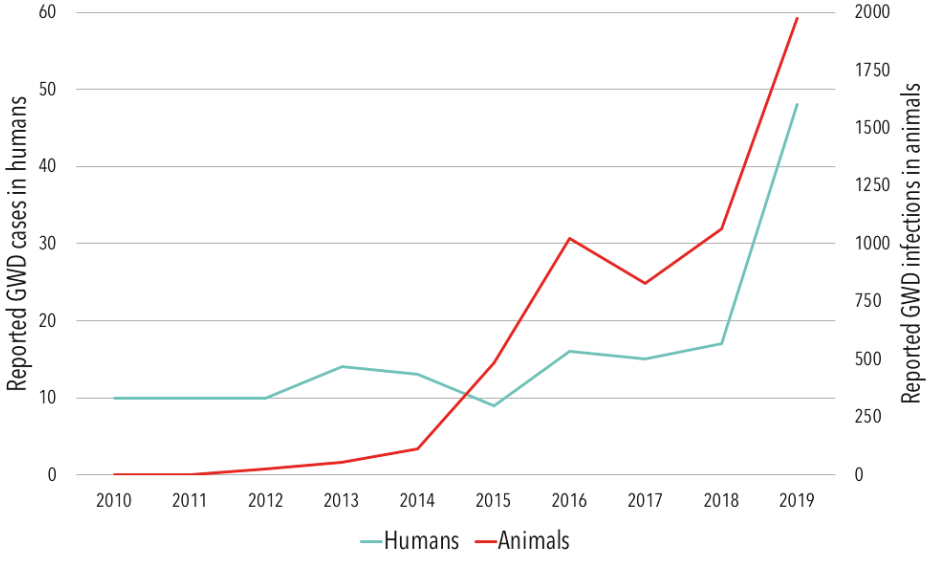

Figure 2. Reported human cases and animal infections of Guinea worm disease in Chad, 2010–2019.

Ethiopia reported one Guinea worm infection in baboons annually from 2013–2015 [28,33], two infections in 2016 [34], four infections in 2017 [35], one infection in 2018 [36], and six infections in 2019 [30]; Ethiopia also reported five infections in cats in 2018 [36]. Mali reported one infection in a cat in 2017 [35], two infections in 2018 [31], and one infection in 2019 [30]. From 2015–2019, Angola and South Sudan each reported one Guinea worm infection in dogs [30,35], and neither has reported infections in other animals.

3.2. Interventions Used to Reduce the Burden of Guinea Worm Disease in Humans and Animal Populations

GWD eradication efforts primarily rely on behavioral- and environmental-based interventions and associated health education campaigns to break the cycle of transmission between humans and the environment [37]. Countries that are endemic for GWD often endorse layered approaches that emphasize the implementation of multiple interventions with the understanding that no single intervention will be perfectly executed [38].

Many GWD eradication programs are based on the provision of improved water sources for human populations [4,18,20,39,40] (Figure 3). Specific interventions include the use of water filtration devices [4,18,20,39,40] and providing clean water from underground sources by hand-dug or bore-hole wells or other similar means [4,18,39,40]. Sand, steel mesh, and finely woven cloth water filtration

devices prevent GWD in humans by removing the copepods from drinking water. Portable pipe filters—straws with a filter inside—allow people to filter their water when traveling or away from home [40]. A more communal solution involves drilling boreholes to provide clean water from underground sources, which protects against other infectious diseases in addition to GWD [40].

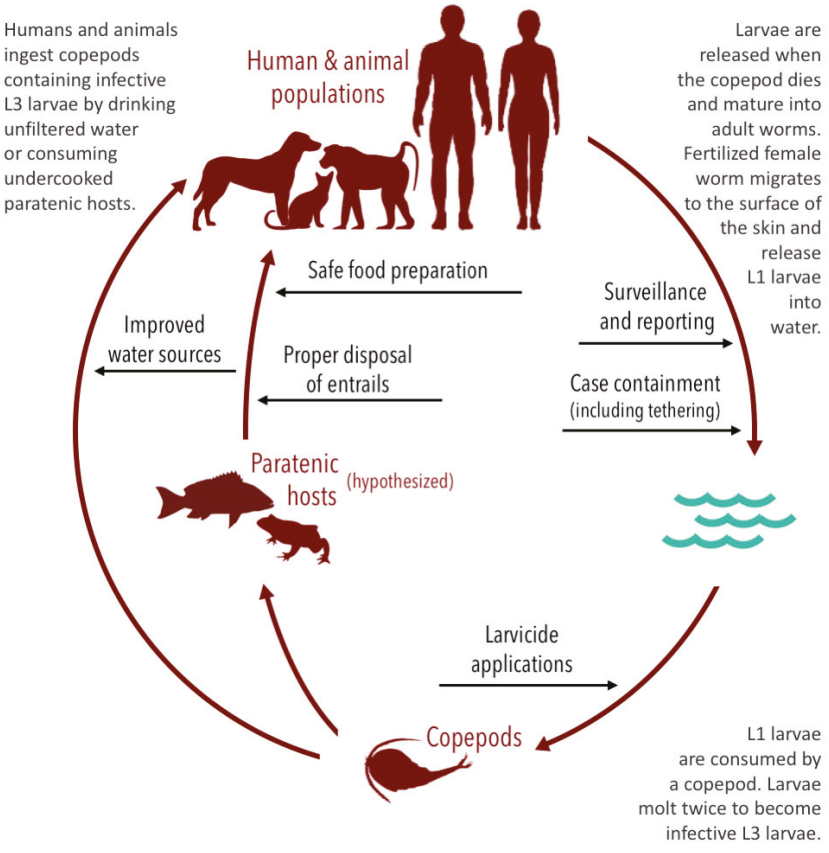

Figure 3. Transmission and life cycle of the Guinea worm with impact points of existing interventions.

To supplement interventions focused on drinking water, eradication campaigns also treat water sources with larvicides to reduce and control vector populations. Temephos is a colorless, odorless, and tasteless larvicide that is harmless to humans, fish, and plants at recommended concentrations, but lethal to the copepods that act as intermediate hosts for Guinea worm [40]. Accordingly, it is frequently used to treat contaminated or potentially contaminated sources of surface water to reduce transmission risk [2–4,12,18,20,40–42]. It may be proactively applied at monthly intervals [4], or reactively following the known contamination of a water source by an infected human or animal within 10–14 days [28].

Challenges related to treating water sources with temephos include the intensity of labor, difficulties in reaching remote areas, difficulties in identifying which surface water sources require treatment, and effectively treating larger or flowing bodies of water (which is generally less successful than treatments of smaller or stagnant bodies) [3,4,18]. Because animals may infect and utilize water sources that are not used by humans, some programs endeavor to map and apply larvicide to hidden water sources in at-risk areas; however, seasonal flooding and the drying of many water sources make this

difficult to track [2,19]. Overall, there is some evidence that suggests vector control interventions are an effective method for suppressing GWD infections via the traditional water-based infection route; in theory, it should also reduce hypothesized paratenic transmission if the infected copepods are killed prior to consumption by paratenic hosts.

Other interventions focus on the containment of human and animal cases before they have an opportunity to enter and contaminate water sources [14,18,20,41,42]. Eradication programs have promoted the tethering of domestic dogs and cats with signs of infection, to prevent them from contaminating water sources once the worms fully emerge; Ethiopia and Chad have piloted preventative tethering of animals without symptoms in villages identified as high-risk [14,41,42]. Eradication programs have provided monetary incentives for complying with tethering protocols; the Ethiopia program has also imposed fines for persons found releasing animals from tethering prematurely [43]. GPS collars have also been piloted to track the movement of baboons [19,36,44], with the goal of monitoring the potential contamination of water sources even in densely forested areas [45].

To increase the sensitivity of surveillance efforts, programs have also introduced cash rewards for reporting suspected cases of GWD in animals and humans that are subsequently confirmed [2,4,18]. This form of active surveillance depends on the rapid investigation of rumors, a robust network of staff and volunteers, and a reliable reporting system [20]. Rewards are generally higher for reporting human cases of GWD and generally increase as countries report fewer human cases. Cash rewards for reporting infections in dogs are occasionally split between the person who reports the dog and the owner of the dog, in exchange for tethering the animal until the worm emerges [35,46–48].

Health education and awareness campaigns are also used to reduce transmission. Campaigns frequently focus on educating people about the transmission cycle, how the infection is acquired, and how it can be prevented [2,18,20,21,40,41]. These efforts are designed to inform people about the aforementioned interventions but have also encouraged other behavioral interventions, such as sufficiently cooking, drying or smoking hypothesized paratenic hosts before eating [2,18,21,41], and burning or burying fish entrails that may contain infected copepods to prevent animals from eating them [2,18,21,41,42]. Campaigns occur in person, through radio and television programming, via printed materials (e.g., posters), and through existing community structures like school systems and houses of religion to achieve maximum reach [48]. These efforts are of importance in endemic communities, neighboring communities, and at-risk non-endemic areas [2].

Some additional interventions have aimed at using medical treatment to prevent or cure infections in dogs. Topical and oral anthelmintics commonly used in dogs as preventives for other nematode infections, such as heartworm, have been tested with limited effects [38,48]. Trials of ivermectin and imidacloprid/moxidectin have not demonstrated effectiveness in preventing or curing infections in dogs and were discontinued [49]; an oral trial with flubendazole is ongoing [38,50]. These recent, limited efforts at incorporating veterinary treatment into human eradication goals may benefit from more laboratory research prior to field trials and, if successful in controlled environments, incorporation into a systemic, long-term interdisciplinary strategy.

4. Discussion

Pathogens with complex transmission cycles that involve multiple hosts can be exceptionally challenging to eliminate and eradicate. The massive mid-21st century campaign to eradicate smallpox was successful because of the comparatively simple, single-host epidemiology of the smallpox virus. Most pathogens of humans are characterized by more complex transmission pathways. Indeed, most infectious diseases of humans have zoonotic origins [51], meaning that, by definition, animals can serve as reservoirs, paratenic or dead-end hosts, or transmitters of the pathogen in question.

GWD, once believed to have been exclusively a disease of humans, has proven capable of infecting other animals. As the lack of zoonotic reservoirs has traditionally been considered a key factor for identifying human diseases with the potential for eradication [52]; this begs the question of whether

GWD should no longer be considered eradicable; whether GWD should be considered eradicable, but only with respect to the disease in humans, and not entirely removed from the ecosystem (i.e., alter the current definition of "eradicated"); or pursue the full eradication of Guinea worm across both humans and animals, and use it as evidence that the previous tenets of "eradicable" are flawed. We advocate for the last of these options and believe that more intensive and holistic interventions and approaches that consider environmental, human, and animal elements as part of an interacting whole are more likely to be successful.

The veterinary profession has targeted prevention and treatment of gastrointestinal worms in dogs and cats as a way not only to keep pets healthy but also to prevent transmission of worms to humans. There is some evidence for success. For instance, higher-income nations often report much lower rates of toxocariasis than lower-income nations [53]. Yet, *Toxocara* seroprevalence and other indicators remain alarmingly high, even in the industrialized world, in part because of earlier misconceptions about the parasite's lifecycle, lack of access to animal care, and practical challenges of treating street dog populations—all challenges similar to those associated with GWD.

Rabies represents another example of successful coordination between human and veterinary health specialists working in lock-step toward the common goal of eliminating rabies in human populations. Many countries have had success with a case management mechanism that spans human and animal healthcare-seeking known as integrated bite case management [54,55]. In addition, countries that have eliminated rabies in people often have dedicated and aggressive canine and feline rabies vaccination programs implemented through legal requirements on pet owners to vaccinate their animals. Indeed, the World Health Organization's strategy for eliminating human rabies in countries where it remains endemic is to eliminate it in dogs through mass vaccination campaigns [56].

While existing interventions for GWD have generally been successful in controlling the disease in human populations, they have been unsuccessful in halting transmission, as infections in animals have recently been detected in all of the remaining endemic countries. The increasing presence of animal infections suggests that improved adherence to existing interventions and/or new interventions targeting animals are necessary [2], especially given that people will likely be at risk so long as the parasite continues to infect animals. Zoonotic diseases, like GWD, may therefore benefit from strategies that explicitly integrate public health, veterinary medicine, animal management, and ecological approaches [57].

A renewed approach to eradicating GWD in affected countries can be achieved through a framework and set of targeted activities that improve disease surveillance in humans and animals; pinpoint the dominant routes of infection in animal hosts; elucidate the burden of disease in animals; determine risk factors for transmission among animals and from animals to humans; and identify practical ways to foster horizontal and multidisciplinary intervention approaches.

The eradication of GWD will require a holistic understanding of the burden of disease and the identification and containment of every case. While daunting, as mentioned earlier, this is necessary because humans and animals are infected by shared populations of Guinea worms, and as a result, humans be at risk for infections so long as the parasite continues to infect animals. This makes disease surveillance, case finding, and case containment a priority [58]. In Chad, a consistent pattern has been the discovery of dog infections with the expansion of active surveillance following identified human cases in new areas. For instance, after 13 human cases were detected in the Moyen-Chari region of Chad between 2010 and 2014, the initiation of active surveillance in 2015 uncovered hundreds of infected dogs in subsequent years; the region reported 409 dog infections in 2018 and 855 dog infections in 2019 [59,60]. A similar discovery may come to pass in Chad's Tandjile region, which only initiated active surveillance in 2019 following sporadic detection of human cases since 2011 [38]. Surveying communities about the year they first noticed worms emerging from dogs—and comparing responses to initiation of active surveillance programs—may provide further insights. Given the relative numbers, it seems more likely that transmission among dogs is driving cases in humans rather than vice-versa, and active surveillance with an equal focus on animal transmission should immediately

follow the detection of human cases in new areas. Because dogs, unlike humans, are not able to report their own infections, exploratory surveillance efforts outside of areas known to be endemic should also be considered. These efforts should include periodic capture and examination of free-ranging and feral dogs in areas known to be endemic, as these animals may perpetuate transmission even when infections in domestic dogs are fully contained.

To this end, identifying practical ways to foster horizontal and multidisciplinary approaches could improve surveillance and control efforts. Coordinating interventions with other human and animal public health initiatives, and leveraging existing One Health efforts, represent opportunities to achieve this goal. For example, in Mali, NTD control efforts use predominantly vertical approaches, and while there is a One Health platform, it is not currently involved in the Guinea worm effort. Integrating surveillance activities in humans and animal populations could ensure more routine activities for both populations also help to ensure that GWD is not reintroduced to areas where it had previously been eliminated. Further, integrating NTD control programs with other health programs has proven to be a practical option for improving accountability, efficiency, and cost-effectiveness [61], and both of the human cases in Angola and Cameroon were initially recognized by healthcare workers who were involved in national immunization campaigns [27,35]. Future work should investigate the cost-effectiveness of current strategies, as well as that of new interventions to help determine which approaches for eradication are most appropriate for areas that remain at risk.

With regard to surveillance in animal populations, integrating Guinea worm and rabies could represent a feasible option, as some countries have robust rabies vaccination programs that could also be used to conduct Guinea worm infection exams. Integrating GWD control efforts with these initiatives may improve the reach, cost-effectiveness, and sustainability of surveillance for dog infections in particular, and may help flag areas endemic for animal transmission for expansion of both active surveillance and control interventions before human cases appear. Different levels of integration could include surveying rabies vaccination teams for reports of rumored dog infections; training rabies teams to identify symptoms, report infections, and inform dog owners about reward money; or, most intensively, providing rabies field staff with sample collection tubes and reward money for identifying confirmed infections in areas beyond the reach of active surveillance. Further, for countries that do not have robust rabies control programs, integrating GWD and rabies control efforts could incentivize collaboration between veterinary and human health departments and correspondingly improve rabies vaccination rates—which would benefit both GWD and rabies control efforts.

Beyond simply detecting infections, understanding relevant risk factors for transmission and how transmission is occurring among dogs, cats, and other animal species would allow for the implementation of timely and more effective control measures. It is possible that animals, and in particular dogs, are exposed to Guinea worm infection by both foodborne and waterborne routes of transmission, or through some combination that amplifies infection in non-human hosts [19]. Although recent research efforts have focused on possible infection of dogs via consumption of raw or undercooked paratenic hosts infected with the parasite [15,20], targeted searches revealed a very low prevalence of infection among frogs and no Guinea worms detected in dissected fish [62]. This may point to waterborne transmission as the dominant route of infection in dogs [17]. This is especially true when comparing against the high infection rates among dogs in the villages in which the fish and frogs were obtained and the frequency of frog consumption among dogs. This hypothesis may also be supported by the observation of year-over-year clusters of worm emergence during short periods of time, due to staggered ingestion of infected paratenic hosts. Examining dog infection line lists may provide further clues and the continued evaluation of transmission routes could help refine or devise interventions [42]. Different approaches may also be required for different animal populations (e.g., domestic versus free-ranging dogs) and it will be important to identify risk factors to inform control efforts [19]. Community surveys about the presence of free-ranging or feral dogs, or wild dogs and cats, in endemic areas could also inform approaches and help to elucidate whether or to what extent these populations play a role in sustaining transmission.

Eradication of any infectious disease requires simple diagnostics, simple preventives and/or treatments, and political will. The growing scientific understanding of the zoonotic capacity of Guinea worm underscores the importance of diagnosis in non-human hosts. Diagnosis is key to enabling proper epidemiological understanding and to inform prevention and treatment decisions. At present, diagnosis in dogs is done in the same manner as for people (i.e., visual detection of the worm at the skin surface). This diagnostic approach allows for detection only at a very late stage of infection. A field-based rapid diagnostic assay such as antibody serology to assess pre-patent infection in dogs could support improved understanding of canine prevalence, and intervention decisions like proactive tethering. Hand-held ultrasound machines, frequently used in equine medicine, could also be used to detect the worms in advance of emergence. Identified infected dogs could then be prioritized by monitoring programs through routine checks for worm emergence. Some of the worms identified on ultrasound could potentially be removed surgically by qualified personnel, such as spay-neuter teams, where such resources are available. Improved diagnostics when combined with an interdisciplinary research and surveillance approach could shift the research paradigm toward a more comprehensive understanding of infection prevalence in other animals. For a less technical approach, routine spot-checks of dogs for signs of worm emergence, either conducted by program staff or owners, may increase early detection and containment of Guinea worms.

Similarly, new tools for environmental surveillance could be useful for targeting limited health education and surveillance resources toward communities and at highest risk. An assay developed to amplify Guinea worm DNA successfully identified larvae from infected copepods present in samples of pond water taken from Chad [63]. While such a tool could potentially shed light on the temporal and spatial distribution of larvae in the environment, a field-informed sampling strategy and a close collaboration with community health workers and water treatment teams would be needed to operationalize the results.

Community responses, participation in, and behavior change stemming from these activities should also be monitored [64], as such considerations will impact overall intervention effectiveness and could allow for eradication programs to tailor intervention strategies to specific sub-populations. For instance, monthly spot-checks of fish entrail burial sites have been conducted in villages under active surveillance in Chad since 2015, with consistent reports of high adherence [27,43,46]. If fish entrail burial is not associated with reductions in animal infections, further analysis of related human and animal behaviors may uncover alternate, more effective interventions, or suggest a different primary route of infection.

Beyond One Health, other aspects that require multisectoral approaches also challenge eradication efforts. For instance, conflict and migration present challenges for containing any infectious disease, but have notably hindered surveillance and control efforts for Guinea worm in both human and animal populations [12]. A new human case in Mali in 2020, the first in four years, may indicate conflict-related surveillance gaps [65]. In the Malian context, insecurity has also limited program operations in regions of the country where dogs are bred and become infected before being transported to other areas [36]. The Guinea Worm Eradication Program of South Sudan is taking action to address some of these challenges by tracking the migratory patterns of individuals associated with cattle camps, which characterized the Guinea worm cases in 2018 [59]. Similar efforts may prove beneficial in Chad, where dogs and humans seasonally migrate to fishing grounds, and where tracking these patterns may help identify new areas of endemicity.

5. Conclusions

The decades-long effort to eradicate GWD has been delayed as a result of contextual challenges but also because infections in non-human animal species are complicating control efforts. While existing strategies and interventions have achieved impressive decreases in human GWD cases, an apparent epidemiological shift occurred over the past decade whereby the largest burden of GWD shifted from human populations to animals. Although this shift likely reflects changes in surveillance and not novel

transmission patterns in other species, it underscores the importance of embracing new approaches if the goal of eradicating GWD is to be realized. Accordingly, a One Health approach that seeks to improve surveillance efforts in human and animal populations, elucidate the burden of disease in animal populations, identify risk factors for transmission among human and animal populations, and foster horizontal and multidisciplinary interventions could represent one way to reach the elusive but attainable goal of eradicating GWD in humans and animals.

Supplementary Materials: The following are available online at http://www.mdpi.com/2414-6366/5/4/159/s1, Table S1: Literature Review Search Strategy. Table S2: Annual and Monthly Reported GWD Case and Infection Data.

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Review

Intestinal Schistosomiasis and Giardiasis Co-Infection in Sub-Saharan Africa: Can a One Health Approach Improve Control of Each Waterborne Parasite Simultaneously?

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Abstract: Both intestinal schistosomiasis and giardiasis are co-endemic throughout many areas of sub-Saharan Africa, significantly impacting the health of millions of children in endemic areas. While giardiasis is not considered a neglected tropical disease (NTD), intestinal schistosomiasis is formally grouped under the NTD umbrella and receives significant advocacy and financial support for large-scale control. Although there are differences in the epidemiology between these two diseases, there are also key similarities that might be exploited within potential integrated control strategies permitting tandem interventions. In this review, we highlight these similarities and discuss opportunities for integrated control of giardiasis in low and middle-income countries where intestinal schistosomiasis is co-endemic. By applying new, advanced methods of disease surveillance, and by improving the provision of water, sanitation and hygiene (WASH) initiatives, (co)infection with intestinal schistosomiasis and/or giardiasis could not only be more effectively controlled but also better understood. In this light, we appraise the suitability of a One Health approach targeting both intestinal schistosomiasis and giardiasis, for if adopted more broadly, transmission of both diseases could be reduced to gain improvements in health and wellbeing.

Keywords: One Health; *Schistosoma mansoni*; *Giardia duodenalis*; water, sanitation and hygiene (WASH); Uganda

1. Introduction

Throughout many tropical and sub-tropical low- and middle-income countries (LMICs) where provision of water, sanitation and hygiene (WASH) infrastructure is inadequate, communities of people are often found to be co-infected with multiple parasitic diseases acquired through ingestion of, or

contact with, contaminated water and food [1,2]. Notably, intestinal schistosomiasis and giardiasis are both waterborne parasitic diseases highly prevalent and co-endemic in these regions.

Intestinal schistosomiasis is a debilitating neglected tropical disease (NTD) caused by infection with parasitic blood flukes of the species *Schistosoma mansoni, S. japonicum, S. intercalatum, S. mekongi* and *S. guineensis* [3]. This disease is highly prevalent throughout many areas of sub-Saharan Africa and locally endemic in some areas of South America and the Caribbean, where the vast majority of cases are caused by infection with *S. mansoni.* Intestinal schistosomiasis compromises the general integrity of the small bowel via egg-induced perforations with associated local and systemic inflammation [3].

Giardiasis, another debilitating but underreported intestinal parasitic disease, is caused by infection with the single-celled eukaryotic diplomonad *Giardia duodenalis*, a flagellated protist [4]. While cosmopolitan in distribution, giardiasis prevalence is particularly high in low and middle-income countries [5,6]. Like intestinal schistosomiasis, giardiasis also compromises the general integrity of the digestive tract. It does this through a major disruption of the gut microbiota, causing a variety of debilitating pathologies such as dehydration and anemia [7,8]. Unlike intestinal schistosomiasis, however, giardiasis is not considered an NTD, although there have been previous discussions proposing its inclusion [9,10].

Whilst there are differences in the transmission biology and epidemiology between both intestinal schistosomiasis and giardiasis, there are also key similarities that might be exploited within potential integrated control strategies, allowing for control of both diseases in tandem. By highlighting these similarities, and by outlining opportunities for integrated control of giardiasis in areas where intestinal schistosomiasis is co-endemic, we aim to diminish the detrimental effects of (co)infection, thereby improving the health and wellbeing of those, particularly children, in endemic areas. To do this, an integrated 'One Health' approach is needed that requires a detailed knowledge of the transmission biology of each parasite, appropriate use of reliable point-of-care (POC) diagnostics, mitigation of environmental and zoonotic transmission through improved WASH infrastructure and effective use of anti-parasitic chemotherapies. Each of these should be carefully considered and applied simultaneously to improve public health outcomes [11–14].

2. Intestinal Schistosomiasis and Giardiasis: Pathology and Epidemiology

Intestinal schistosomiasis disproportionally afflicts school-aged children between the ages of six and fifteen years old, where pathology can be both acute and chronic [3]. As based on 'classic' age-infection profiles and measured using faecal egg counts, the intensity of infection typically begins to decline in late adolescence while morbidity associated with *S. mansoni*, such as multi-organ fibrosis, accumulates. This decline in egg-patent prevalence is due to a variety of factors such as partial-immunity to infection, notwithstanding extensive fibrosis of the bowel itself which can occlude egg exit sites, giving rise to granulomatous masses known as intestinal 'bilharzomas' [15,16].

Pathologies associated with intestinal schistosomiasis occur primarily as a result of the body's response to the copious number of eggs produced by female adult worms inhabiting the mesenteric veins surrounding the intestines. Rather than being passed in stool (or occasionally in urine) to perpetuate the parasites lifecycle, a large proportion of eggs will instead become sequestered throughout the venous bloodstream of the intestinal and hepatoportal tracts. Many eggs will then enter into general venous circulation and subsequently become lodged in other major organs. Once eggs become trapped, for example in the intestinal wall and/or liver sinuses, a range of clinical systemic and organ-specific morbidity ensues, including acute abdominal pain, stunted growth, environmental enteropathy, presence of faecal occult blood and overt hepato/splenomegaly [17,18].

Human giardiasis is caused by infection with *G. duodenalis* (syn. *Giardia intestinalis*, *Giardia lamblia*). While *G. duodenalis* is the only human-infecting *Giardia* species, eight distinct evolutionary assemblages based on multi-locus genotyping, named A through H, are known to exist [19]. Of these eight, the vast majority of human infections are caused by assemblages A and B, although human infections with assemblages C, D, E and F do also occur, albeit rarely [19,20].

Unlike the distribution of *S. mansoni*, which is intrinsically linked to its *Biomphalaria* spp. intermediate freshwater snail hosts, the distribution of *G. duodenalis* is truly cosmopolitan. Giardiasis prevalence in humans is particularly high; however, in LMICs lacking access to clean, safe drinking water and associated WASH infrastructures, including many areas of sub-Saharan Africa and South America, where *S. mansoni* is also endemic [5,6]. A notable feature of giardiasis, in humans, can be asymptomatic infections, although acute and/or chronic and debilitating pathologies owing to infection are well described. These include diarrhoea, dehydration, malabsorption, tropical enteropathy, stunted growth, impaired cognitive development, anaemia and chronic fatigue [7,8]. The primary cause of these pathologies is a major disruption to the gut microbiota, a complex community of symbiotic microbes responsible for vitamin production, nutrient absorption and regulation of lipid metabolism, brought about through *G. duodenalis* invading, inhabiting and multiplying within the intestinal tract [21–23]. Importantly, severe morbidity is most often observed in certain high-risk groups including children, the elderly, those with physical disabilities and the immunocompromised [24,25].

2.1. Common Modes of Environmental Contamination

A major factor linking the transmission of both intestinal schistosomiasis and giardiasis is their transfaecal environmental contamination routes via the excretion of schistosome eggs (*S. mansoni*) or cysts (*G. duodenalis*) into a viable body of freshwater. Although waterborne transmission is not required for *G. duodenalis* to complete its life cycle, and while not all *S. mansoni* eggs or *G. duodenalis* cysts will successfully reach a viable freshwater habitat, in a disease-endemic setting, many environmental water bodies will undergo some extent of direct or indirect faecal contamination with both parasites (Figure 1) [3,4,26,27]. Indirect faecal contamination of freshwater can occur, for example, while bathing, through infected stools deposited on the banks of rivers and ponds being washed into these waters by heavy rains or floods, through overflowing pit latrines, and possibly through animals such as cattle walking through sites of defecation and transporting faecal matter to bodies of water on their hooves [28,29]. *S. mansoni* eggs can survive up to approximately eight days in the stool post-defecation and before reaching freshwater, whereas *G. duodenalis* cysts can survive up to eight weeks in the environment [4,30] (Table 1).

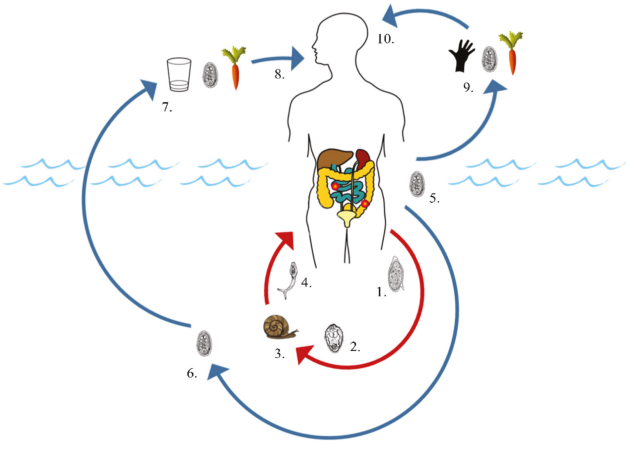

Figure 1. Transmission routes of *S. mansoni* (red, 1–4) and *G. duodenalis* (blue, 5–8 and 9–10). Both parasites are transmitted through faecal contamination of freshwater (*S. mansoni*: 1, *G. duodenalis*: 5). Infection with *S. mansoni* primarily occurs through cercarial penetration of the skin upon contact with contaminated water (4). Waterborne infection with *G. duodenalis* occurs through consumption of contaminated water or foods washed with contaminated water (7–8). Infection with *G. duodenalis* can also occur through consumption of contaminated foods or unwashed hands (9–10). Adapted from [26,27].

Once exposed to freshwater, *S. mansoni* eggs (Figure 1, (1)) will hatch to release free-swimming ciliated miracidia (Figure 1, (2)) that will then employ a range of morphological adaptations and host-seeking behaviours to locate and penetrate the soft tissues of its freshwater snail intermediate host, *Biomphalaria* spp. (Figure 1, (3)) [31–33]. Miracidia are ephemeral, living only a short period of time, typically less than six hours, before dying as their glycogen energy reserves are exhausted (Table 1).

Miracidia that successfully invade a suitable intermediate snail host metamorphose into mother sporocysts, which, in turn, produce daughter sporocysts. These daughter sporocysts then differentiate upon sporogenesis, producing numerous cercariae that are shed from the snail approximately one month after initial invasion by the miracidium (Figure 1, (4)). Once established, cercarial production and shedding from *Biomphalaria* spp. snail hosts occur daily and typically continue over the remainder of the snails' lives. Over the course of an infected snail's life, tens of thousands of cercariae can be liberated [3,34].

Shed cercariae will then go on to infect humans and other mammalian definitive hosts, primarily through cutaneous penetration (Figure 1, (4)), although infection may also occur through penetration of the buccal cavity when consuming contaminated water [30]. Like miracidia, cercariae are ephemeral in freshwater as their glycogen energy reserves are quickly depleted, lasting no longer than three days (Table 1). In addition, survival of both miracidia and cercariae is highly dependent on favourable biotic and abiotic environmental conditions. Freshwater too high in salinity or too polluted, for example, can prevent the hatching of eggs into miracidia and can be lethal to both miracidia and cercariae [32,33,35,36].

G. duodenalis has a direct, faecal–oral life cycle that can be completed either through waterborne transmission when consuming water contaminated with cysts (or foods/utensils washed using contaminated water without sufficient soaps) (Figure 1, (5–8)), or through the consumption of contaminated foods or unwashed hands (Figure 1, (9–10)) [4,37]. Unlike *S. mansoni*, which will asexually reproduce within an intermediate host, and although *Giardia* cysts may survive and even accumulate within certain filter-feeding freshwater invertebrates (for example, oysters), *G. duodenalis* does not require an intermediate host for transmission [38]. Cysts passed in the stool are, however, extremely resilient and can remain viable within the stool or in freshwater for up to eight weeks after excretion (Table 1) [4,37,39].

Table 1. Survival and viability of *S. mansoni* eggs and *G. duodenalis* cysts in the environment post-defecation.

Species	Life-Stage	Survival Post-Defecation in the Stool or in Freshwater	Reference(s)
S. mansoni	Eggs	~8 days in stool prior to reaching freshwater	[30]
	Miracidia	<6 h in freshwater	[31–33]
	Cercariae	~1–3 days in freshwater	[32,33,35,36]
G. duodenalis (Assemblages A and B)	Cysts	Up to eight weeks in stool or in freshwater	[4,37,39]

Maintained transmission of intestinal schistosomiasis is therefore dependent on the continued contamination of freshwater and continued exposure to contaminated/infested water, whereas giardiasis transmission is heavily exacerbated by the continued contamination of freshwater and continued consumption of contaminated water. There are a variety of ways in which an individual can be exposed to and infected with both parasites, for example, when water is used for consumption, sanitation purposes, income generation from fishing or farming and/or recreation [40,41]. As such, transmission of both diseases is particularly high in impoverished areas lacking adequate WASH infrastructures, such as access to functional pit latrines and clean drinking water, as well as behavioural impediments in those unaware of how to avoid contamination and infection [42,43].

2.2. Transmission via Non-Human Hosts

The transmission of both intestinal schistosomiasis and giardiasis is also exacerbated by a range of non-human definitive hosts acting as either major or minor reservoirs of infection, although the precise extent to which these hosts contribute to human transmission is not fully understood (Table 2). Further to the significant health and economic impact of infection with African schistosomes and/or *Giardia* on, for example livestock, animal reservoirs of both parasites also pose challenges in controlling and reducing human transmission as each parasite follows similar routes of infection, contamination and, ultimately, environmental transmission via non-human hosts [5,44,45].

Table 2. Primary reservoir hosts of *S. mansoni* and *G. duodenalis* (assemblages A-H). '+' denotes known primary reservoir host; '-' denotes no known primary reservoir host.

Species	Humans	Non-Human Primates	Ruminants	Rodents	Other Mammals	Fish	References
S. mansoni	+	+	-	+	-	-	[46,47]
G. duodenalis (assemblage A)	+	+	+	+	+	+	[20,48–51]
G. duodenalis (assemblage B)	+	+	+	+	+	+	[20,48–50]
G. duodenalis (assemblage C)	- *	-	+	-	+	-	[19,20]
G. duodenalis (assemblage D)	- *	-	+	-	+	-	[19,20]
G. duodenalis (assemblage E)	- *	-	+	-	+	-	[19,20]
G. duodenalis (assemblage F)	- *	-	-	-	+	-	[19,20]
G. duodenalis (assemblage G)	-	-	-	+	-	-	[19,20]
G. duodenalis (assemblage H)	-	-	-	-	+	-	[19,20]

* Human infection possible but rarely observed [20].

To reduce human transmission effectively, animal reservoirs and the degree to which they contribute to and maintain disease transmission must therefore be carefully considered when developing, implementing and monitoring any disease control strategies. Moreover, particular attention is needed on those animal hosts able to reintroduce parasites into viable bodies of freshwater following prior control or elimination campaigns [52]. Limiting contact of cattle with freshwater, for example, as well as limiting run-off from fields on which cattle manure has been spread and disposing of animal waste away from bodies of freshwater are known to reduce transmission of *Giardia*, as well as non-human infecting *Schistosoma* species [53,54]. Doing so, however, can be extremely challenging to implement and maintain through time.

In light of recent findings, additional consideration should also be given to the potential emergence of schistosome hybrids and their impact on schistosomiasis transmission [55–57]. *Schistosoma mansoni*, for example, can form hybrids with rodent-infecting species *S. rodhani*, which have been observed along the shoreline of Lake Victoria, one of the Great East African lakes. However, *S. rodhaini* appears exclusive to rodents, together with the *S. mansoni-rodhaini* hybrids, but with many gaps in routine surveillance this appraisal may be incomplete [58,59]. Uniquely among trematodes, schistosomes are dioecious, and so adult worms form inter-species copulatory pairs which facilitate permissive introgression(s). In nature, pre- and post-zygotic reproductive isolating barriers, such as host specificity, anatomical site of infection, geographical distribution, mating preference, worm competition and snail incompatibility, are thought to prevent prolific inter-species admixture. Recently, however, and owing to advanced methods of molecular analysis on schistosome larval stages from snail-intermediate and mammalian-definitive hosts, surprising inter-species hybrid forms are now being identified in several endemic African countries [58]. Such hybrids, resulting from interactions between human- and

animal-infecting species, not only raise concerns about zoonotic transmission, but also the expanded host ranges and increased transmission potential acquired through heritable traits [59].

Changes to natural landscapes can readily lead to the formation of new freshwater bodies, snail habitats and multi-host transmission sites, breaking down the ecological barriers between species and leading to further inter-species interactions. Although the full impact that these hybridization events may have on human disease epidemiology and disease pathology is currently unknown, hybridization certainly suggests that future schistosomiasis control may warrant an expanded One Health approach with more tailored interventions specific to local settings and schistosome epidemiology [56,58,60].

3. Intestinal Schistosomiasis and Giardiasis: Surveillance and Control

Highlighting these key similarities in disease transmission, biology and epidemiology between *S. mansoni* and *Giardia* presents clear opportunities for integrated surveillance and control of both diseases, particularly with regard to disease diagnosis, surveillance and control.

3.1. Diagnosis: Parasitological, Immunological and Molecular Methods

Owing to its low cost, portability and high specificity, light microscopy for the detection of *S. mansoni* eggs in faecal samples is widely used during disease surveillance programmes to detect infection with *S. mansoni* in sub-Saharan Africa [61]. Using microscopy, routine parasitological surveillance to assess endemicity and prevalence of intestinal schistosomiasis, as well as other intestinal helminth infections, in a community is typically carried out via the Kato-Katz technique using faecal samples provided by school-aged children [62]. While inexpensive and portable, the sensitivity of Kato-Katz is, however, severely reduced in low-intensity infections, hampering its use in areas of low disease endemicity or in areas having undergone successful disease control intervention(s) [63–65].

Giardiasis cannot be reliably detected using the Kato-Katz method, and so alternative methods, such as formalin/ether concentration techniques, flotation techniques or immunofluorescent antibody microscopy, are needed [11,66–68]. Unfortunately, however, none of these techniques are straightforward or inexpensive to carry out under rural field conditions, particularly as it has been reported that formalin/ether concentration techniques have a higher sensitivity in detecting infection with *S. mansoni* than the more field-deployable Kato-Katz technique [69,70]. Moreover, these techniques can also often be insufficiently sensitive to reliably detect giardiasis infection [68].

For these reasons, a variety of immunological and molecular diagnostic assays with improved sensitivity in detecting both *S. mansoni* and *Giardia* infection using non-invasive urine and faecal samples have been developed (Table 3).

Table 3. Overview of primary diagnostic assays to detect infection with *Schistosoma mansoni* and *Giardia duodenalis*.

Species	Direct Diagnosis	Antigen Detection	Molecular Diagnosis
S. mansoni	Identification of ova in concentrated faecal smear via Kato-Katz technique [62,71]	Detection of circulating cathodic antigen (CCA) or circulating anodic antigen (CAA) in urine samples using ELISA or lateral-flow test strips [72,73]	Detection and amplification of species-specific DNA in faecal samples using PCR/qPCR [74], (LAMP and RPA assays have also been developed [71,75–77]).
G. duodenalis	Identification of cysts in concentrated faecal smear via formalin/ether concentration techniques, flotation techniques or immunofluorescent antibody microscopy [68]	Detection of species-specific antigens in faecal samples using ELISA or lateral-flow test strips [11,78–80]	Detection and amplification of species-specific DNA in faecal samples using PCR/qPCR [78], (LAMP and RPA assays have also been developed [81–83]).

Though highly sensitive, immunoassays such as the enzyme-linked immunosorbent assay (ELISA) and molecular assays such as PCR/qPCR require specialist laboratory infrastructure seldom available in disease-endemic areas, preventing their use at POC [84,85]. As such, several rapid and field-deployable RDTs have also been developed to detect trace levels of parasite-derived antigens and parasite-derived DNA in urine and faecal samples. Some examples include straightforward lateral-flow dipsticks to

detect *S. mansoni* circulating cathodic antigen (CCA) in urine samples and *G. duodenalis* (with or without *Cryptosporidium* spp.) cyst antigen in faecal samples, as well as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) assays to detect species-specific *Schistosoma*- and *Giardia*-derived DNA in urine and faecal samples [11,64,85].

While POC-RDTs have many advantages over light-microscopy, microscopy remains less financially expensive to carry out and so it is the favoured method of diagnosis during routine monitoring and control programmes with only limited financial resources available [68,75,78]. In addition, and though promising, assays such as LAMP and RPA to detect species-specific parasite DNA currently require further assessment and validation before their upscaled and routine use in such control programmes [81,85]. Nevertheless, continued development, assessment and validation of POC-RDTs is widely advocated as affordable and sensitive point-of-care diagnostics, capable of detecting low levels of infection within individuals able to maintain disease transmission, are sorely needed [86]. Given these challenges in reliably detecting infection using human samples, particularly in low-endemicity settings, alternative methods of detecting and monitoring disease transmission within endemic foci, such as parasite host surveillance and use of environmental DNA (eDNA), have also been explored.

3.2. Exploring the One Health InterFace with Increased Host Surveillance

Intermediate hosts and definitive reservoir hosts, such as *Biomphalaria* freshwater snails (*S. mansoni*) and rodents or cattle (*S. mansoni* and *G. duodenalis*, respectively), offer an alternative means of detecting and monitoring disease transmission in areas where detecting transmission through human diagnosis may be unreliable [87]. Collecting freshwater snails capable of transmitting schistosomes and carrying out shedding analyses to assess cercarial emergence, for example, may help identify active transmission sites [87,88]. This approach, however, can also be unreliable, as very few snails are typically found to be shedding cercariae [89].

For this reason, highly sensitive molecular xenomonitoring approaches to detect *Schistosoma* DNA in snail host tissues have also been developed and assessed [90]. Using PCR to detect *Schistosoma* DNA within snail hosts, for example, can identify prepatent infections and is not affected by diurnal fluctuations in cercarial shedding in the same way that cercarial shedding is; allowing a more reliable assessment of schistosome presence in a given locality than shedding analyses can allow. An added advantage of molecular xenomonitoring by use of PCR is the ability to genotype parasite and snail DNA, providing valuable opportunities to better understand disease transmission and molecular epidemiology, such as more reliable species identification of human-infecting cercariae and snail intermediate hosts than can be achieved using morphological analysis and the detection of *Schistosoma* hybridisation events [55]. In addition, collecting and screening freshwater snail hosts for the presence of parasite DNA can be more straightforward and more lucrative than collecting and screening human faecal samples for parasite DNA [90]. Currently, however, mass collection and molecular screening of freshwater snail hosts using PCR remains logistically, technically and financially demanding, and so development of a high-throughput methodology, possibly incorporating use of rapid and POC DNA amplification technologies such as LAMP or RPA, or pooling of snail samples, should also be further explored and assessed [91,92].

Similarly, molecular detection of *S. mansoni* and *G. duodenalis* DNA in DNA extracted from faeces collected from definitive reservoir hosts capable of perpetuating transmission, such as rodents (*S. mansoni*) and cattle (*Giardia*), has also been used to monitor disease transmission with success [49,58]. An added benefit of collecting and analysing faecal samples in this way is that this method also provides an opportunity to assess and better understand wild-type *Schistosoma* hybridisation events and zoonotic transmission of human-infecting *G. duodenalis* through genotyping [55,93,94]. Again, however, this approach too requires significant financial and technological resources. As such, it is unlikely to be widely integrated into control programmes undertaken in low-resource areas such as

rural regions of sub-Saharan Africa without further development and use of field-deployable DNA amplification technologies.

3.3. Detecting Parasitic Contamination through Water Sampling and by Environmental DNA (eDNA) Analysis

Extensive screening of environmental water samples for contamination with *Giardia* cysts using the United States Environmental Protection Agency method 1623 (US-EPA method 1623) has been carried out in many areas of the world, such as the USA [95]. Through collection and filtration of water samples, protozoan cysts can be detected and quantified, allowing viable transmission sites to be identified [96]. Although straightforward to carry out, this method does not differentiate between morphologically identical *Giardia* assemblages A-H and so identification of human-infecting *Giardia* transmission sites, specifically, is not possible. As such, revised methods of detecting and monitoring assemblage-specific *Giardia*-contaminated water sources, such as through detection and genotypic analysis of parasite-derived eDNA, have been developed.

Assessing and monitoring disease transmission within a given focus through the detection of parasite-derived eDNA rather than, or in conjunction with, using human bodily samples, has been explored with respect to a range of waterborne pathogens, including both schistosomiasis and giardiasis [97–99]. Dependence of both parasites on freshwater provides an ideal target for sample collection and assessment using PCR/qPCR, LAMP or RPA assays [83,100,101]. In addition, collection of water samples to detect eDNA derived from *Schistosoma* freshwater snail hosts to identify and monitor the presence of snail species capable of transmitting infection within a given waterbody has also been assessed [102,103].

Again, though promising, the upscaled and routine use of molecular assays to detect parasite- and/or parasite host-derived eDNA remains beyond the financial reach of most LMIC control programmes and too requires further methodological development, assessment and validation. Nevertheless, continued development of this approach to better understand the potential of eDNA as an effective monitoring tool and to reduce associated financial costs has been encouraged [75]. In particular, and like molecular xenomonitoring approaches, the monitoring of eDNA to identify disease transmission may prove extremely useful in areas of low disease endemicity where identifying infection in individual patients may be challenging.

3.4. A Case Example of Co-Infection and Morbidity Surveillance in Uganda

Given these key similarities in disease transmission biology and co-endemicity of both intestinal schistosomiasis and giardiasis throughout much of sub-Saharan Africa, co-infection with both parasites is likely commonplace, yet only little formal attention has been given towards co-surveillance of both diseases. This is despite each parasite potentially influencing reciprocal infection susceptibilities and disease-associated pathologies, before and after anti-parasitic treatment(s). As an example, it is possible that *S. mansoni* egg-induced perforations to the bowel with associated mucosal bleeding, inflammation and bacterial translocation may influence an individual's susceptibility to chronic *Giardia* infection. The extent to which this occurs, however, is currently unknown.

This lack of attention on co-infection and co-surveillance may be, in part, due to an unfortunate division within parasitology that often siloes macro-parasite (helminth) and micro-parasite (protist) research. Though sparse, recent epidemiological studies are now beginning to shed more detailed light on the prevalence of co-infection of intestinal schistosomiasis and giardiasis, with detection of associated morbidities, throughout rural areas of sub-Saharan Africa [11,104,105]. A suitable example arises from two recent studies assessing co-infection in school-aged children along the shoreline of Lake Albert, Uganda, which, despite ongoing preventive chemotherapy for intestinal schistosomiasis, can still be considered hyper-endemic for *S. mansoni* today (Figure 2) [88]. Here, initial infection with *S. mansoni* occurs very soon after birth, with all ages vulnerable to infection and chronic disease [106].

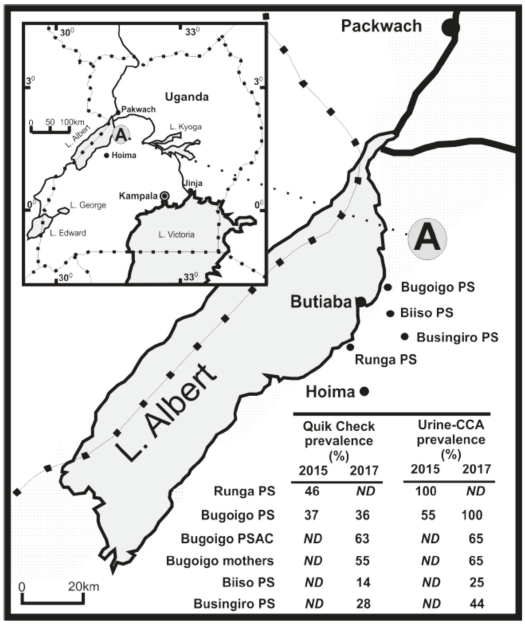

Figure 2. High prevalence of intestinal schistosomiasis (assessed using urine-CCA POC-RDT) and giardiasis co-infection (assessed using Quik Chek POC-RDT (TechLab, USA)) in school-aged children across multiple communities along the shoreline of Lake Albert, Uganda in 2015 and 2017 [8].

Beginning in 2015, Al-Shehri et al. [8] conducted a novel attempt to integrate surveillance for intestinal schistosomiasis, giardiasis and malaria using available POC rapid diagnostic tests (RDTs) combined with later real-time qPCR analysis of stool and finger-prick collected blood with parasite-specific TaqMan DNA® probes. This was the first attempt to quantify giardiasis with the POC Quik Chek RDT (TechLab, USA), finding 42% of children attending Runga and Bugoigo primary schools to be positive (Figure 2). Upon qPCR analysis of ethanol preserved stool using an 18S rDNA *Giardia*-specific TaqMan® probe, up to 87.0% of children were found excreting *Giardia* DNA. Notably, the prevalence of heavy infection by real-time PCR (Ct ≤ 19) was 19.5% and strongly associated with Quik-Chek RDTs, as well as postively correlated with increasing intensities of egg-patent schistosomiasis and host anaemia [11].

Giardia species assemblages present were also later identified and characterised with specific triose phosphate isomerase (TPI) Taqman® probes and by sequence characterisation of the *β*-giardin gene [107]. While less sensitive than the 18S rDNA assay, general prevalence by TPI probes was 52%, with prevalence by taxon assemblage of 8% (assemblage A), 36% (assemblage B) and 8% co-infection (A and B assemblages), and while assemblage B was dominant across the sample, proportions of assemblages A and B, and co-infections thereof, varied by school and by age of child. Mixed infections were particularly common at Runga school and in children aged 6 and under. Most importantly, infection with assemblage B was associated with underweight children. The presence of each assemblage was also confirmed by sequence analysis of the *β*-giardin gene finding sub-assemblage AII and further genetic diversity within assemblage B; also of note was the absence cryptosporidiosis, another pertinent water-borne disease, concurrently detectable by the same Quik Chek RDT.

To assess any changes through time, a repeat epidemiological survey was undertaken in 2017 which included reinspection of Bugoigo school and expanded point-of-care testing with Quik Chek (Figure 2). The prevalence of giardiasis at Bugoigo primary school was shown to be identical with a third of children examined positive by Quik Chek, with even higher local prevalence in pre-school-age

children (63%) and their mothers (55%), good evidence for pervasive nature of giardiasis across all ages. Away from the lake at Biiso and Busingiro, the prevalence of giardiasis and intestinal schistosomiasis declined, suggesting that the risk of infection is perhaps higher on the lake shoreline. This study also attempted to evaluate a new POC-RPA RDT onsite, as well as a pilot assessment of giardiasis in local livestock and companion animals [81]. Ultimately, the RPA assay did not perform as well as expected, being in need for further optimisation of stool DNA extraction protocols.

Further to human-surveillance, screening for *S. mansoni* transmission has also taken place along the same shoreline by collection and cercarial shedding of *Biomphalaria* freshwater snail hosts with success [88,108]. Future surveillance should also be carried out through molecular xenomonitoring of collected snails (and so omitting the need for shedding analyses), collection and screening of non-human definitive hosts to identify *S. mansoni* and *G. duodenalis* DNA and through collection and screening of surface water samples to identify *S. mansoni* and *G. duodenalis* eDNA.

3.5. Access to Treatment and Large-Scale Campaigns

In areas where schistosomiasis transmission is identified, preventive chemotherapy through repeated mass drug administration (MDA) of the donated anthelmintic drug praziquantel (40 mg/kg body weight) is the principal strategy for disease control [109]. Because the highest burden of infection is typically seen in children and young adolescents, MDA is customarily carried out in schools, but aims to limit overall transmission within a community through a reduced human reservoir of infection while also reducing overall disease morbidity [110]. Though praziquantel's mechanism of action is not currently fully understood, significant reductions in disease prevalence and morbidity have been seen globally since MDA programmes began in 2001 [111,112]. Reinfection of schistosomiasis following treatment is, however, commonplace owing to communities' reliance on freshwater, and so MDA must be repeated annually or biannually, depending on disease prevalence, to achieve a sustained impact.

Severe adverse effects are seen only very rarely when distributing praziquantel, making it well suited for mass distribution. Praziquantel, however, typically does not achieve 100% infection clearance primarily because dosing is usually based only on height and so does not account for differences in body mass. As a result, treatment success varies between individuals meaning many are still able to continue maintaining transmission [113]. In addition, and while local school systems provide a viable means of mass-distributing praziquantel, important human reservoirs of infection, including pre-school-aged children and adults, typically remain untreated [106,114].

The need for repeated annual or biannual distribution of MDA in this way has also raised regular concerns about the development of praziquantel resistance in schistosomes; particularly as there is currently no known efficacious alternative treatment to replace praziquantel if *Schistosoma* populations were to become more drug-tolerant or resistant [115,116]. A significant reduction in praziquantel efficacy, identified by a decreased reduction in *Schistosoma* egg output from infected individuals pre- and post-praziquantel treatment, has already been reported in *S. mansoni* populations in many communities across sub-Saharan Africa that have undergone repeated rounds of MDA [113]. This reduced efficacy may be a direct result of selection pressure placed on schistosomes during repeated and prolonged MDA campaigns, highlighting an urgent need to consider alternative methods of disease control outside of MDA.

A variety of drugs can be used to treat giardiasis [117,118]. Of these, metronidazole is the most predominantly used and most thoroughly studied owing to its straightforward oral administration and relatively low price. Like with schistosomiasis, reinfection with giardiasis is also commonplace; however, repeated mass drug administration to alleviate giardiasis transmission is not seen as a feasible strategy because the drug is not currently involved in any donation scheme, severe adverse effects of treatment are often seen, and metronidazole has only limited efficacy in clearing infection [117]. As an example, it has been reported that just one course of treatment has only an approximately 60% clearance rate, and so repeated treatment is needed to significantly clear infection [119]. Repeated treatment, however, not only significantly increases the likelihood of severe adverse events but is

difficult to carry out during MDA campaigns [5]. In addition, the potential emergence of giardiasis resistance to treatment with metronidazole has also recently been reported, and while alternative and more efficacious chemotherapies, such as tinidazole exist, these can also cause adverse events [117–119]. Albendazole, a broad-spectrum and efficacious anthelmintic treatment used in MDA campaigns to reduce transmission of soil-transmitted helminth and some filarial nematode infections, can also be used to treat giardiasis [117,120,121]. To significantly reduce *Giardia* infection, however, a minimum dosage of 200–800 mg/day albendazole is needed for at least three concurrent days which, again, is difficult to carry out in the context of MDA campaigns and, by having limited donated stocks, also diminishes albendazole availability for anti-helminth control programmes [117,118,122].

Though treatment of schistosomiasis and giardiasis using praziquantel and metronidazole are important components of disease control, aligning treatment of both diseases in tandem during control campaigns may be challenging, owing to differences in drug type, dosages and treatment courses. In addition, as neither drug can safely and reliably achieve a 100% clearance rate using just one dosage, it is now widely accepted that alternative methods of control to reduce transmission and overall prevalence must be implemented alongside treatment campaigns if disease elimination targets are to be met. One such example is the implementation of WASH initiatives in communities where both diseases are endemic. The extent to which WASH provision, when used in conjunction with MDA, can successfully reduce schistosomiasis transmission is now beginning to be understood, and although only minimal data has been reported on the impact of WASH provision on giardiasis transmission in sub-Saharan Africa, it is widely assumed that improved WASH infrastructure would help significantly reduce giardiasis transmission [1,123,124].

3.6. Water, Sanitation and Hygeine (WASH)

WASH provision and infrastructure is extremely inadequate throughout many areas of rural sub-Saharan Africa [125]. In 2012, the World Health Assembly (WHA) formally advocated for the integration of WASH provision and education initiatives into amenable NTD control and elimination programmes; subsequently publishing guidance on ways in which these can be integrated [126,127]. Since, much attention has been given towards how WASH initiatives can be tailored for use, specifically, in schistosomiasis control programmes and the impact such initiatives have had when used in tandem with routine strategies such as preventive chemotherapy [2,30,128,129].

WASH initiatives relevant to schistosomiasis control, such as the adequate provision of safe drinking water, fully functional and properly maintained pit latrines and improved community hygiene education, effectively reduce disease transmission by minimising the direct and indirect contamination of freshwater by infected individuals and animals, by reducing contact with/consumption of infectious waters by human and non-human hosts and by helping communities better understand human and non-human disease transmission [30,130] (Figure 3).

Like intestinal schistosomiasis, giardiasis is widely prevalent throughout many rural and impoverished regions of sub-Saharan Africa and is intrinsically linked to contact with contaminated and unsafe water in areas lacking adequate water, sanitation and hygiene (WASH) infrastructure [4,41]. Giardiasis, however, receives only relatively little attention with regard to disease control, surveillance and elimination throughout sub-Saharan Africa.

As an example, despite numerous clear advantages of implementing WASH initiatives on reducing schistosomiasis transmission and despite these key similarities between schistosomiasis and giardiasis with regard to disease transmission biology, surprisingly little attention has been given to the impact of improved WASH provision and education on giardiasis transmission in sub-Saharan Africa [43]. This oversight presents not only a missed opportunity with regard to better understanding, and reducing, giardiasis transmission and its associated pathological impact on some of the world's most disadvantaged communities, but also presents the question: why is giardiasis ignored in intestinal schistosomiasis monitoring and control programmes?

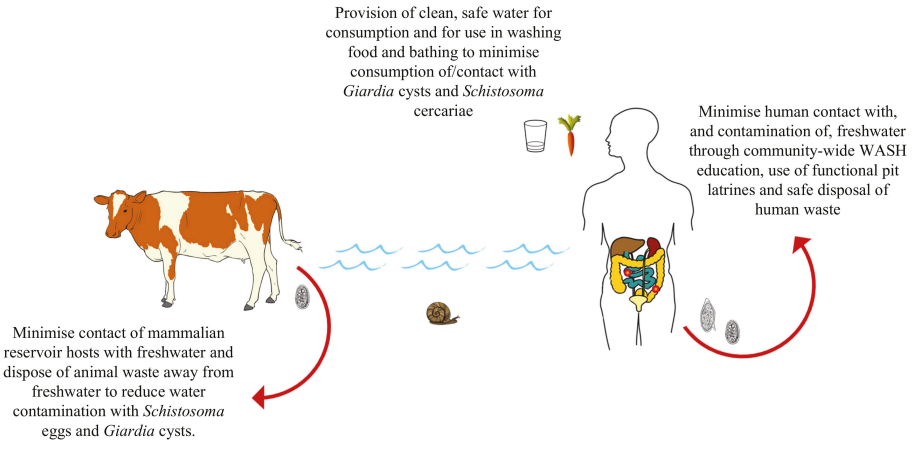

Figure 3. Examples of water, sanitation and hygiene (WASH) initiatives implemented to prevent the contamination of freshwater with *S. mansoni* eggs and *G. duodenalis* cysts, as well as to prevent contact with and consumption of contaminated water. Adapted from [26,27].

4. Intestinal Schistosomiasis and Giardiasis: Towards a One Health Approach

Research funding opportunities for NTDs are limited when compared to those for other diseases such as human immunodeficiency virus (HIV), malaria or tuberculosis [131]. One way in which the impact of NTD control programmes can be significantly increased, however, is by appropriate integration with other disease surveillance, control, research and policy efforts. Successful examples of this integrated One Health approach can be seen when integrating lymphatic filariasis surveillance and elimination efforts into malaria elimination programmes, as well as by integrating soil-transmitted helminth and schistosomiasis control and elimination efforts [128,132–135].

In keeping with this integrated One Health approach, here, we propose a variety of ways in which the transmission of, and pathologies associated with, co-infection of intestinal schistosomiasis and giardiasis can be better understood, monitored and reduced via the integration of giardiasis control efforts into existing schistosomiasis control programmes. These include:

- Integrating screening of giardiasis endemicity and infection prevalence into existing schistosomiasis control programmes by using stool samples used for diagnosing infection with *S. mansoni*, and other intestinal parasites, to also record and report levels of *Giardia* infection in school-aged children. This can be conducted with POC-RDTs such as the Quik Chek immunoassay or using PCR/qPCR. In addition, the continued development, assessment and application of sensitive and straightforward POC-RDTs able to detect low-levels of infection in asymptomatic individuals capable of maintaining transmission of both parasites, such as the RPA, is encouraged.
- Further development and application of sensitive molecular assays to detect trace levels of species/assemblage-specific parasite DNA within freshwater snail intermediate hosts of human-infecting *Schistosoma*, and in faecal samples from non-human animal definitive hosts of both diseases. Further development and application of sensitive molecular assays to detect trace levels of species-/assemblage-specific parasite DNA from human-infecting *Schistosoma* cercariae and *Giardia* cysts in water samples easily collected from viable transmission sites is also encouraged.
- The upscaled provision of water, sanitation and hygiene (WASH) infrastructure and education initiatives to communities afflicted by both schistosomiasis and giardiasis to reduce environmental contamination events and to reduce contact with/consumption of contaminated water, simultaneously reducing transmission of both diseases.

- Monitoring *Giardia* disease prevalence and associated morbidities in tandem with schistosomiasis surveillance in school-aged children following any control programme intervention to better understand how giardiasis transmission and related pathologies can be reduced.
- An increased focus on understanding how the transmission of intestinal schistosomiasis and giardiasis, as well as immune responses and morbidities related to both diseases, interact and are potentially exacerbated by co-infection.

5. Conclusions

Here, we have highlighted various potential opportunities to improve the health and wellbeing of individuals in low- and middle-income countries where intestinal schistosomiasis and giardiasis are co-endemic by exploiting key similarities between both diseases with regard to disease transmission biology, epidemiology, surveillance and control. In addition, future steps needed to develop and implement an integrated, One Health approach for intestinal schistosomiasis and giardiasis co-infection surveillance, control and elimination strategies, are also outlined. In adopting this One Health approach, and by integrating giardiasis surveillance, control and elimination efforts into existing schistosomiasis elimination programmes, not only can the debilitating pathological impacts of intestinal schistosomiasis/giardiasis co-infection be better understood, but a reduction in co-infection and concurrent reduction in disease-related morbidities experienced by the world's most disadvantaged communities can also be achieved.

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Abbreviations

eDNA	environmental DNA
ELISA	enzyme-linked immunosorbent assay
FGS	female genital schistosomiasis
LAMP	loop-mediated isothermal reaction
LMIC	lower-middle-income countries
MDA	mass drug administration
NTD	neglected tropical disease
PCR	polymerase chain reaction
POC	point-of-care
qPCR	quantitative polymerase chain reaction
RDT	rapid diagnostic test
RPA	recombinase polymerase amplification
Th1	T-helper 1
Th2	T-helper 2
WASH	water, sanitation and hygiene
WHA	world health assembly

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Review

Insights into the Control and Management of Human and Bovine African Trypanosomiasis in Zambia between 2009 and 2019—A Review

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Abstract: Tsetse transmitted trypanosomiasis is a fatal disease commonly known as *Nagana* in cattle and sleeping sickness in humans. The disease threatens food security and has severe economic impact in Africa including most parts of Zambia. The level of effectiveness of commonly used African trypanosomiasis control methods has been reported in several studies. However, there have been no review studies on African trypanosomiasis control and management conducted in the context of One Health. This paper therefore seeks to fill this knowledge gap. A review of studies that have been conducted on African trypanosomiasis in Zambia between 2009 and 2019, with a focus on the control and management of trypanosomiasis was conducted. A total of 2238 articles were screened, with application of the search engines PubMed, PubMed Central and One Search. Out of these articles, 18 matched the required criteria and constituted the basis for the paper. An in-depth analysis of the 18 articles was conducted to identify knowledge gaps and evidence for best practices. Findings from this review provide stakeholders and health workers with a basis for prioritisation of African trypanosomiasis as an important neglected disease in Zambia and for formulation of One Health strategies for better control and/or management of the disease.

Keywords: trypanosomiasis; control; management; One Health; Zambia

1. Introduction

African trypanosomiasis is endemic to Sub-Saharan Africa and continues to threaten human health and food security. African trypanosomiasis has been a major draw-back to agriculture and economic development in affected countries, with annual losses in agricultural gross domestic product estimated at USD 4.7 billion [1,2]. The current strategy of the Zambian government to preserve natural resources and create state protected National Parks (NPs) and Game Management Areas (GMAs) has led to an expansion of wildlife populations that serve as long term reservoirs for African trypanosomiasis, and also to an increase in the population of tsetse flies that transmit the disease [3]. At the same time, increase in human population density and the changing climate, particularly rainfall patterns, have forced people (and their livestock) to migrate into these GMAs in search of fertile land for farming. Such uncontrolled migration of people into protected areas has brought about changes in

land use patterns that threaten to alter tsetse habitat quality and patterns of African trypanosomiasis transmission due to increased tsetse–human and tsetse–livestock contacts [4–6].

Tourists visiting NPs and GMAs have not been spared from risks of Human African Trypanosomiasis (HAT) infections occurring through transmission from wildlife reservoir hosts [6]. Despite cases of HAT reported from tourists after their visit to Zambia's NPs, [7,8] there are gaps in protecting tourists and international travellers from tsetse and HAT. Some tour operators have taken it upon themselves to undertake some interventions, particularly in the form of tsetse control, aimed at reducing the risk of HAT infection among tourists visiting their facilities. Such limited interventions produce very limited levels of effectiveness or success, considering that such interventions need to cover considerable large proportions of the affected areas and as such require the collective input of many key stakeholders [9].

Tsetse flies are found in about 37% of Zambia's land area, and it is estimated that the prevalence of African animal trypanosomiasis (AAT) in cattle ranges from 1% to 90% depending on the area [8]. Most of the affected areas are located in rural remote parts of the country and as such the direct negative impacts of the trypanosomiasis problem occur in communities that live in these areas. These impacts include serious economic consequences such as reduced livestock productivity and mortality and the high cost of treating affected livestock [10,11].

In Zambia, AAT has been managed through constant use of trypanocides by individual livestock farmers, while treatment and/or management of the disease in humans has been negatively affected by several factors that include late case detection that tends to result in tragic consequences (death) associated with adverse effects of the administered drugs in the late stage [12,13]. The Zambian government has generally made some notable strides in the control of African trypanosomiasis particularly through tsetse control. However, the government's inability to put in place active surveillance systems, and the lack of adequate resources to effectively sustain control efforts, have contributed to the limitation of success associated with tsetse re-invasion and resurgence of African trypanosomiasis in areas where the disease had earlier been brought under control. In the case of HAT, lack of active surveillance systems has historically hindered progress towards the goal of eliminating African trypanosomiasis as a public health problem in Zambia [14,15].

The period between 2009 and 2019 has seen a significant number of undertakings focused largely on the parasite, transmission and epidemiology of African trypanosomiasis. However, no systematic review of the literature has been conducted on the control and management of African trypanosomiasis in Zambia particularly from a One Health perspective. This review seeks to address this knowledge gap.

2. Materials and Methods

With a focus on studies conducted on HAT and AAT control in Zambia, a systematic review (Figure A1 in Appendix A) of published data was undertaken. Using three searches with three categories of key words, a cumulative total of 2238 peer reviewed articles were identified in December 2019 from the following three search engines: PubMed, PubMed Central and One Search. One Search was used because it has a wider research area while PubMed was used because it is more aligned with veterinary sciences. Using the following key words: trypanosomiasis AND control AND management AND One Health AND Zambia, 610 articles were identified. In addition, two independent searches were performed using key words: trypanosomiasis AND control AND Zambia (995 articles identified), trypanosomiasis AND control AND One Health AND Zambia (633 articles identified). Duplicate articles were removed after which remaining articles were screened by title and abstract to assess the relevance of documents. Articles related to biochemical and biological developments in tsetse and African trypanosomiasis diagnostic assays were excluded from the review as most of the articles were focused on the trypanosome agent rather than management and control. Inclusion criteria were as follows: (i) studies conducted on the control and management of African trypanosomiasis in Zambia, (ii) related to One Health, (iii) related to African trypanosomiasis diagnostic methods, (iv) published in English only, and (v) published between January 2009 and December 2019. A final full text screening

from the search conducted left 18 articles that met the inclusion criteria for the review (Table A1 in Appendix B). To support and supplement data from articles included in the review, published and unpublished government records and reports related to tsetse and African trypanosomiasis control for the same period were also referenced.

This review was conducted as part of a PhD project with ethical clearances from James Cook University (H7226 and A2498), Zambian Ethics Committee (Ref. No. 2018-Oct-001) and research approval from the Zambia National Health Research Authority.

3. Results

Based on the analysis of publications included in this review (Table A1 in Appendix B), results indicate that various trypanosome species circulate within a wide and diverse host community in Zambia [4]. The presence of the tsetse fly has facilitated the circulation of the parasite in the ecosystem [16]. Movement of people has led to the development of a new wildlife/livestock/human interface [4,17] *T. congolense* and *T. vivax* are the major causes of clinical AAT in cattle with low packed cell volume (PCV) usually an indicator of infection [18–20]. Infections with *T.b.r* in domestic animals remained a significant indicator that domestic animals could be reservoirs of HAT. Findings show that the impact of AAT is highest in cattle with dogs becoming a potential reservoir host for the human disease [16,21].

Current diagnostic methods used in Zambia do not conform to what is now thought to be the best practice [18,22,23]. Diagnosis of African trypanosomiasis remains a challenge in endemic areas of Zambia due to low staffing levels and non-functional laboratories [24,25].

Food security for communities living in tsetse-infested areas has continued to be negatively impacted [26]. The impact of AAT can be reduced through use of trypanocides and application of insecticide to control tsetse flies. Cattle farmers living in African trypanosomiasis-endemic areas and GMAs have resorted to drastic use of trypanocides to combat the disease [11]. African trypanosomiasis control in Zambia has been focused on cattle and not humans [26], with nothing published on the control and management of the disease in other domestic animals. Wildlife trypanosomiasis hosts pose a risk to communities and tourists living near or in national parks and game reserves [2,7,8].

Despite Zambia having had three major African trypanosomiasis control programmes (aerial spraying, insecticide treated targets and trypanocide drug use), the country has recorded several disease re-occurrences in areas where control was once undertaken. New cases are being reported in new areas while some old foci are disappearing [27,28]. Despite the evidence of the occurrence of African trypanosomiasis in both humans and livestock and the challenges faced by communities living in tsetse-infested areas, there is no One Health approach to control the disease [2,16,26].

A weak health system is in place for the management of HAT. Knowledge of HAT management among health workers is unsatisfactory [24]. A wide diversity of control programmes are available but lack government support [15,24,25,28]. Stakeholders in Zambia have competing views and beliefs regarding tsetse and African trypanosomiasis control, which is critical in developing a One Health approach for the control in both HAT and AAT. Environmentalists believe tsetse flies help keep environments wild and natural by stopping farmers encroaching protected areas. Agriculturalists feel that such moves have contributed to increased poverty as farmers are kept away from protected areas that are tsetse-infested [26].

4. Discussion

The Luangwa and Zambezi river basins support high densities of tsetse flies and wildlife reservoirs of African trypanosomiasis [4]. This review of tsetse and African trypanosomiasis studies undertaken in Zambia clearly indicates that most of these studies have been undertaken from or along the peripherals of the two river basins. With an estimated 37% of Zambia's land area tsetse-infested, the risk of African trypanosomiasis infection for people and livestock living in the tsetse-infested areas in the country cannot therefore be overemphasised [11].

An assessment by the World Health Organization (WHO) indicated that HAT usually affected people whose occupations took them into tsetse-infested areas. Categories of people so affected include among others: small scale farmers, workers under wildlife services, tsetse control workers, poachers, honey gatherers and fishermen [13]. Increased human populations and thus increased demand for land for agriculture continues to force people and their livestock into tsetse-infested areas in search for fertile land. Migration of people with their livestock into tsetse-infested areas, as highlighted in this review, has resulted in changes in the epidemiology of African trypanosomiasis. Livestock rearing in these tsetse-infested areas has thus eroded the diverse ecosystems and led to the development of a new kind of wildlife/livestock/human interface with domestic animals acting as potential link for trypanosome exchange [4,14,29].

The risk of HAT infection in travellers to national parks and game reserves has however not received much attention. Despite reported cases of HAT from tourists after visiting tsetse-infested areas [7,8], there are currently no deliberate interventions in place to protect international travellers from tsetse flies and HAT. In Zambia, most tsetse interventions have been focused in areas with potential for livestock production, with little synchronisation with human intervention programmes [30,31]

Currently, African trypanosomiasis control in humans relies on early diagnosis and treatment. However, challenges in HAT diagnosis in rural settings of Zambia has hindered progress to the control of the disease. Most diagnostic health centres in rural Zambia depend on microscopy for diagnosis. Despite the low sensitivity associated with microscopy, the test remains the gold standard for both HAT and AAT diagnosis because it is affordable. However, the low sensitivity exhibited by microscopy makes it difficult to determine disease incidences, especially in cases where parasitaemia is low, thus stressing the need to improve field diagnosis of African trypanosomiasis [16,19,22,30].

Recent developments of molecular tools such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) for detecting trypanosomiasis has provided hope for improving field diagnosis which may lead to eliminating African trypanosomiasis [32–34]. LAMP has been proven to be more sensitive than microscopy in detecting infections of *T. brucei* and *T. vivax* as compared to *T. congolense.* Such findings indicate the importance of LAMP in epidemiological studies related to HAT rather than AAT. The simplicity and sensitivity of LAMP makes it an ideal diagnostic tool for HAT [16,23,30]. On the other hand, multispecies PCR can identify several species of trypanosomes in a single PCR reaction, thus reducing the cost of molecular diagnosis. The main advantage of molecular tools over microscopy is for epidemiological studies and to identify different trypanosome species [35–37] other than point of care diagnostic tools. Limited support from relevant authorities has negatively impacted on the use of molecular methods in Zambia. Most molecular laboratory consumables cannot be sourced locally, therefore, procurement of consumables has remained a challenge even for institutions that have implemented the use of molecular tools.

For continued efforts to control African trypanosomiasis infections, there is a need to establish strong active and passive surveillance systems in African trypanosomiasis focal point areas. In the absence of diagnostic centres as seen in most rural settings of Zambia, departments of Health and Veterinary services can share resources, diagnostic capacities and personnel for improved case detection, treatment and control of African trypanosomiasis and other zoonotic diseases. Future control efforts for HAT may also consider simultaneous control of the disease in livestock and wildlife reservoirs as a One Health approach [25,26].

Meanwhile, lack of political commitment to sustain tsetse and African trypanosomiasis control programmes [38] has pushed livestock farmers to constant use of trypanocides. The study conducted by Mbewe et al. [12] confirms that livestock farmers living in GMAs or near NPs where tsetse challenge is high have resorted to constant trypanocide use to protect their livestock, which may have serious consequences related to trypanosome resistance to trypanocides [39]. Treatment of infected animals may seem to be the best option for most livestock farmers, but it may tend to be unsustainable and costly in the long run as AAT is largely a herd health problem (Tsetse and Trypanosomiasis section strategic plan 2020, Zambia-unpublished government record). Unfortunately, most farmers living in

tsetse-infested areas treat their animals based on clinical signs and symptoms due to lack of access to laboratories and regular surveys from local veterinarians. In this case, most infections remain in their livestock populations and may be responsible for sustaining sporadic African trypanosomiasis incidences within their communities [40].

Earlier studies by Simukoko et al. [18], indicate that livestock treatment with trypanocides is dependent on seasonal variations of tsetse populations and the risk of AAT infection. Such findings indicate the need for tsetse and AAT control programmes to be focused on seasonal differences in the risk of AAT infection when tsetse challenge is highest. Key stakeholders can therefore use such findings to link to biological characteristics of the tsetse vector in developing cost effective and sustainable control programmes during periods of highest challenge [18,41]. From a travel medicine perspective, such findings also highlight risk periods for travellers.

Increased focus on communicable and non-communicable disease management has pushed African trypanosomiasis off the government's priority list. There is a need to holistically quantify the impact and cost of African trypanosomiasis again in the context of disease prioritisation within Zambia and similarly affected countries. Lack of sustainable control programmes and the absence of a national surveillance and control programme for African trypanosomiasis among others, have impacted negatively on control efforts [24–26,38]. Breaking down barriers between social and natural scientists will help in developing a more holistic One Health approach to control tsetse flies and African trypanosomiasis in Zambia. Lessons learnt from past tsetse and African trypanosomiasis control operations can be useful in developing future cost effective and sustainable control programmes as well as informing health practitioners as to the risks travellers face in visiting these travel destinations and the in-country health support system available to them.

5. Recommendations

It is recommended that:

- Work is done to evaluate and identify African trypanosomiasis control programmes that are cost effective and sustainable in the regions where they are applied.
- Data on biological characteristics of tsetse and seasonal differences in African trypanosomiasis infection risk be considered when developing tsetse and trypanosomiasis control programmes in Zambia.
- More robust field diagnostic procedures for African trypanosomiasis be developed that consider the environmental, capacity and infrastructure constraints of working in countries like Zambia.
- Line Ministries consider sharing resources in order to improve diagnosis and treatment of African trypanosomiasis and other zoonotic diseases.
- A One Health approach be considered for the control of African trypanosomiasis in humans, livestock, wildlife and tsetse flies.

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Abbreviations

PCV	Packed Cell Volume
HAT	Human African Trypanosomiasis
PCR	Polymerase Chain Reaction;
LAMP	Loop Mediated Isothermal Amplification;
WBC	White Blood Cell;
CSF	Cerebrospinal Fluid;
GMA	Game Management Area;
CAT	Canine Animal Trypanosomiasis;
SRA	human Serum Resistance Associated;
RIME	Repetitive Insertion Mobile Element;
CON2-LAMP	specific primer targeting the 18Rrna gene of *T. congolense*; RHC, Rural Health Centre;
DNA	Deoxyribonucleic acid;
FAO	Food and Agriculture Organization RTTCP, Regional Tsetse and Trypanosomiasis Control Programme;
SAS/SAT	Sequential Aerial Spraying;
ITT	Insecticide Treated Targets and Traps;
TRY	trypanocidal drugs;
PATTEC	Pan African Tsetse and Trypanosomiasis Eradication Campaign;
AAT	Animal African Trypanosomiasis;
ITC	Insecticide Treated Cattle

Appendix A

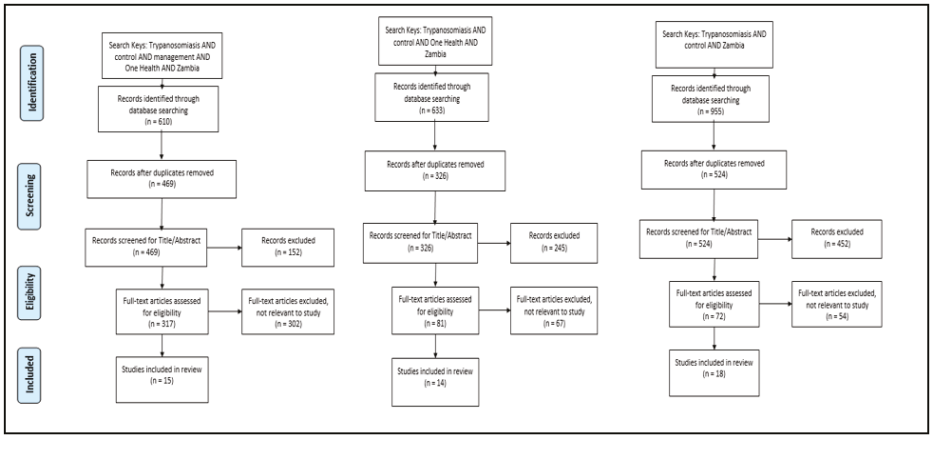

Figure A1. PRISMA diagrams showing three categories of searches conducted and selection criteria of literature included in the review.

Appendix B

Table A1. Articles meeting selection criteria on trypanosomiasis control in Zambia between January 2009 and December 2019 and a summary of key findings.

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Simukoko et al., 2011 [16]	To assess the monthly risk of bovine trypanosomiasis in cattle kept in tsetse-infested eastern plateau of Zambia.	Longitudinal study of bovine trypanosomiasis incidence in cattle	Eighty-five herds of cattle that grazed together were selected for a 19-month follow-up study	-The risk of trypanosome infection varied significantly between months with the higher risk recorded between December and February. -PCVs of infected and un-infected cattle did not differ significantly -*Trypanosoma congolense* and *T. vivax* were detected in 92.3% and 4.5% of the infected cattle, respectively. Mixed infections were detected in 3.2% of positive samples. -Overall, 155 infections were detected using PCR while microscopy detected 85 infections.	1. More effort in optimizing Animal African Trypanosomiasis (AAT) control during periods of highest challenges. 2. Accuracy of AAT incidence using parasitological diagnosis stresses need for more sensitive diagnostic tools to improve field diagnosis.
(Mwanakasale and Songolo, 2011) [28]	-To identify districts in Zambia that were still reporting cases of Human African Trypanosomiasis (HAT). -To compare the occurrence of HAT cases before and after year 2000.	-Cross sectional survey of districts located close to national parks. -Literature review of occurrence of HAT in Zambia in the 1960s to 1990s.	-Conducted in nine provinces of Zambia except for Lusaka district. -Used google search, PubMed and world health organisation HINARI access to obtain data on HAT occurrence. Only articles with data on HAT distribution, epidemics, treatments and control of HAT before 2000 were reviewed.	-Chama, Mpika and Chipata districts were still reporting HAT cases. Seven districts that used to report HAT no longer had cases after January 2000. -All surveyed districts had no existing tsetse control programs. -In all surveyed health institutions, giemsa stain thick smear microscopy was the routine diagnostic method to detect HAT. Only Chilonga mission hospital used microhaematocrit centrifuge method to detect HAT. -Six of the surveyed hospitals had stocks of suramin but none had melarsoprol. -Findings from literature survey show a significant difference in HAT reporting foci from 1960s to 1990s and 2000 to 2007 with some old foci disappearing whilst new ones emerged or re-emerged	1. Districts reporting HAT 2008 to date 2. Data on Agriculture practices between 2000 to 2007 and compared with 1960s to 1990s to confirm if agriculture practices may have contributed to reduced tsetse flies in previously tsetse-infested areas and thus the drastic reduction of HAT cases. 3. Current data on human activities occurring in game management areas (GMAs) as they may be responsible for persistent HAT transmission and tsetse-human contacts. 4. Human animal contacts as animals may carry trypanosomes with them. 5. Poaching as game destruction was once used to eliminate wildlife reservoirs. 6. Under diagnosing of HAT due to increased focus on management of HIV/AIDS and malaria.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Anderson et al., 2011) [4]	To characterise the nature of the reservoir community for trypanosomiasis in the absence of influence from domesticated hosts	A cross-sectional survey of trypanosome prevalence in wildlife hosts. Conducted in the Luangwa valley from 2005 to 2007	A total of 418 wild animals were examined for the presence of trypanosomes	-Overall prevalence in all species was 13.9% with infection likely to be detected in waterbuck, lion, kudu and bushbuck, respectively. -Bushbuck indicated to be important hosts for *T. brucei s.l* with bushbuck, greater Kudu, and Lion to be important hosts for *T. congolense* while *T. vivax* was frequently detected in waterbuck. -*T. b. rhodesiense* were first identified in African buffalo and *T. brucei s.l* in leopard -First use multispecies PCR for the diagnosis of samples collected from free ranging wildlife which offers improved diagnostic specificity and sensitivity compared to traditional techniques. -Results indicated the ability of trypanosomes to survive in a wide variety of wildlife hosts.	1. Tsetse blood meal preference was identified as a risk factor for trypanosome infection. 2. Difficulties in sampling wildlife and method used to sample in this study limited ability to investigate age as a risk factor in trypanosome infection 3. Infection of *T. b. rhodesiense* in buffalo raises concern on possibility of infection been established in cattle populations not far from sampling area i.e., Mambwe district of the eastern province of Zambia. This is because buffalos move over large distances with potential to disseminate infection to other species. 4. Trypanosome reservoir in wildlife hosts maybe wider that estimated in this study 5. Influx of people with their livestock and land use may have an impact on the epidemiology of African trypanosomiasis.
(Namangala et al., 2012) [21]	To evaluate the performance of repetitive insertion mobile element (RIME)–loop mediated isothermal amplification (LAMP) and human serum resistance associated (SRA)–LAMP against microscopy in HAT diagnosis	Case study	Four male patients from Luangwa and Zambezi river basins	-Both RIME-LAMP and SRA-LAMP were able to detect *T. b. rhodesiense* in patients' blood and in cerebrospinal fluid (CSF). -LAMP results correlated with microscopy results but they do not confirm the standard staging criteria using microscopy and white blood cell (WBC) in CSF.	1. Need for a detailed study with larger sample size to evaluate potential of LAMP to be used as a bedside diagnostic test for HAT and for making therapeutic decisions. 2. Need for both active and passive surveillance of HAT and community sensitisation in HAT old foci.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Namangala et al., 2013) [41]	To evaluate the performance of LAMP against microscopy to detect CAT in exotic dogs	Cross sectional survey of trypanosomiasis in exotic dogs	Six exotic dogs naturally infected with trypanosomes from Zambia's South Luangwa National Park and Chiawa GMA.	-Results indicated first report of canine animal trypanosomiasis (CAT) in Zambia -All cases initially diagnosed by microscopy and later confirmed by LAMP, showing good correlation between the two methods. -Three dogs reported infection with *T. congolense* according to CON2-LAMP -All SRA-LAMP positive cases were also RIME-LAMP positive indicating similar sensitivity.	1. Further investigation on SRA gene isolated from two dogs in this communication. 2. Scanty parasitaemia sometimes pose challenges caused by weak fluorescence signal thus need to quantify the fluorescence intensity and consider samples to be positive after subtracting the background fluorescence of the negative control. 3. Dogs as potential source of HAT infections 4. Need to investigate performance of LAMP in CAT diagnosis among locally bred dogs in tsetse-infested GMAs and National parks.
(Mwanakasale et al., 2013) [23]	To assess current health delivery system in the management of HAT.	Cross sectional survey of health institutions using structured questionnaires	Nine health institutions from Mpika district of Zambia were involved in the study	-The general knowledge on HAT of health staff from surveyed health institutions was unsatisfactory for proper management of the disease -Study revealed gross understaffing of essential staff to clinically diagnose and manage HAT-No staff from the surveyed institutions had received specific training on HAT diagnosis and treatment. -There was only one treatment centre (Chilonga mission hospital) from the surveyed health institutions -Erratic supply of trypanocides at the only treatment centre in the district -Only 2 of the surveyed institutions has functional laboratories with qualified personnel. Both institutions used less-sensitive methods to diagnose HAT -Distances between rural health centres (RHCs) and treatment centres and non-availability of transport to ferry suspected HAT patients.	1. Need for refresher courses to be conducted every two years for health personnel in districts at risk of HAT transmission in Zambia. 2. Need for awareness on HAT for health policy makers so that they understand the need for refresher courses and trainings on disease management. 3. Need to motivate in kind health staff at the frontline of identifying suspected cases and encourage them to refer such cases to diagnostic and treatment centres 4. Need to establish Mpika district hospital as an additional treatment centre to decongest Chilonga mission hospital and improve health service delivery at both hospitals.Ministry of health to ensure that drugs for both stages of HAT are always in stock. 5. Need for Ministry of health to equip and capacitate health institutions with laboratories and personnel as well as more sensitive diagnostic tools. 6. Need for a proper referral system for HAT suspected cases to diagnostic treatment to ensure they reach their designated centres.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Lisulo et al., 2014) [19]	To evaluate the performance of LAMP in determining trypanosome prevalence in indigenous dogs.	Cross sectional survey of Canine African Trypanosomiasis	A total of 237 indigenous dogs from 47 villages within five chiefdoms of Mambwe district of Zambia	-Fourteen cases of trypanosomes were detected using microscopy. -LAMP detected an additional 6 cases indicating higher sensitivity and specificity than microscopy. -Adult dogs were more likely to acquire CAT as they are involved in hunting. -CAT was significantly related to corneal opacity -Dogs are potential links for trypanosome exchange between livestock and humans.	1. Diagnostic accuracy of LAMP against microscopy suggested that its use in CAT diagnosis could improve disease management in African trypanosomiasis in endemic areas. 2. Results from study can trigger a One Health approach towards control of HAT through disease intervention in livestock. 3. Need for continuous surveillance of African trypanosomiasis in tsetse-infested regions using more user friendly and sensitive tests such as LAMP. 4. Need to sensitise locals in GMAs potential dangers of keeping dogs that are left to scavenge without receiving veterinary services. 5. Dogs may harbour other zoonoses apart from *T. b. rhodesiense* with potential serious implications to human health.
(Nyimba et al., 2015) [30]	To determine the prevalence and species distribution of caprine trypanosomiasis	Cross-sectional cluster survey of AAT in goats	Overall, 422 goats from Kalomo and Sinazongwe districts of Southern province of Zambia	-One goat was found infected on microscopy while 100 goats reported positive for AAT on LAMP. -Infection rate for Sinazongwe district was 22.4% while that for Kalomo district was 24.7% -*Trypanosoma brucei*, *T. vivax* and *T. congolense* were detected in 82.0%, 31.0% and 23.0% of the infected goats, respectively. Mixed infections were detected in 33.0% of positive samples. -Study results indicate the re-emergence of AAT in study areas were aerial spraying was once conducted by the government.	1. Need for improved staffing to enhance disease prevention and containment. 2. Need for refresher courses for frontline Veterinary staff in order to improve service delivery. 3. Need for sustainable control operations to avoid tsetse re-invasions and re-occurrence of disease in areas where control was once a success story.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Laohasinnarong et al., 2015) [14]	To examine the presence of different trypanosome species in cattle, goats and tsetse using a combination of microscopy, PCR and LAMP	Cross sectional survey of trypanosomes in cattle, goats and tsetse flies.	In total, 243 cattle, 36 goats and 546 tsetse flies were examined for the presence of trypanosomes. Study conducted from Petauke, Chama and Isoka districts of Zambia.	-Microscopy exhibited relatively low sensitivity than PCR and LAMP -There was poor agreement among test methods. For instance, failure of PCR and LAMP to detect microscopically positive samples. -KIN PCR was found to be sensitive for detecting *T. congolense* -TviCatL-PCR and PFL-LAMP were better for detecting *T. Vivax* and *T. b. rhodesiense*, respectively. -The presence of *T. b. rhodesiense* in tsetse samples indicates its ability to take blood meal from multiple hosts (wildlife, humans and domestic animals), facilitating the circulation of the parasite in the ecosystem. -Infection in cattle and goats was highest with *T. congolense* and least with *T. vivax*	1. Need to establish if trypanosome DNA detected from cattle, goats and tsetse were active infections or residual DNA from dead trypanosomes picked from blood meals or treated animals. 2. Need for a One Health approach towards the control of HAT through disease intervention in livestock, wildlife and tsetse.
(Mulenga et al., 2015) [22]	To investigate health personnel's and health centre's capacity to diagnose Human African trypanosomiasis	Cross sectional survey using structured questionnaires.	A sample of 101 health personnel drawn from 12 and nine health centres from Chama and Mambwe districts, respectively	-Staffing levels from both districts were extremely low with most health centres manned by one trained staff -Staff had basic knowledge to identify HAT with staff from Chama districts more likely to identify a case compared to their Mambwe counterparts. -Only Chama district had functional laboratories. Most health centres surveyed reported frequent use of rapid test kits for diagnosing mainly malaria parasites thus reducing diagnosis of other blood parasites that can be detected by microscopy including HAT.	1. Need for authorities to train and post more health staff in rural areas and to come up with deliberate policies that provide incentives to attract and motivate health workers in rural areas 2. Need for capacity building and refresher trainings for health staff with regards to HAT diagnosis. 3. Need for health centres located in HAT foci to be equipped with at least microscopes to enable them more easily identify cases when they occur. Further, referral or district hospitals can also be equipped with more sensitive laboratory tools like PCR and LAMP. 4. Need for HAT national surveillance and control programmes to be enhanced.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Mbewe et al., 2015b) [10]	To examine how socio-economic and environmental factors are associated with adherence to the recommended guidelines on trypanocide use	Cross sectional survey using a structured questionnaire.	Farmers interviewed from five veterinary camps from Itezhi tezhi district of central province of Zambia	-Of the interviewed farmers, 25.6% adhered to FAO guidelines on trypanocide use; (i) reducing the number of treatments on whole herd up to a maximum of four times in a year by integrating drug usage with other control measures and (ii) avoiding exposure of the whole parasite population to the drug by limiting treatments to individual sick animals. -None of the socio-economic factors (age, education, cattle herd size, competence in trypanocide use and access to extension on trypanocide use) were associated with a farmer's adherence to FAO guidelines. -Low adherence to recommended FAO guidelines on trypanocide use was associated with the location of crush pen, whether in GMA or not, as an environmental factor. Farmers in GMAs were less likely to adhere to FAO guidelines than those in non-GMA.	1. Need for an integrated approach of measure to control AAT in the GMA of Itezhi tezhi to lessen overuse of trypanocides by farmers. 2. Need to investigate if household income may influence farmer's adherence to FAO guidelines of trypanocide use as defined in this study. 3. Need to investigate if household income may influence control of vector borne diseases.
(Mbewe et al., 2015a) [20]	-To investigate the prevalence of animal trypanosomiasis in anaemic cattle	Cross sectional survey of AAT in cattle	A total of 564 Anaemic cattle from Itezhi tezhi district of Zambia	-Out of 564 cattle screened, 58 (10.3 %) had anaemia. PCR-RFLP results showed that 17 (29.3 %) anaemic cattle were positive for pathogenic trypanosomes compared to 1 (1.7 %) on parasitological examination using thick smears. -Infections were caused by *Trypanosoma congolense* and *Trypanosoma vivax*.	1. Need to investigate other anaemia causing factors in animal trypanosomiasis endemic areas of Itezhi tezhi district of Zambia.
(Grant et al., 2015) [24]	-To examine the narratives on African trypanosomiasis in Zambian policy. -To explore relationships between human, animal and environmental sectors	Case study of key informant interviews	Twenty participants from international organisations, research organisations and local activists.	-Environmentalists believed tsetse stop farmers encroaching protected areas thus keeping areas natural and wild. -Increased poverty because tsetse keeps farmers away from productive areas. -The Zambian government has other diseases of priority other than African trypanosomiasis and does not have funds to keep areas tsetse free. -Major focus of African trypanosomiasis control is emphasised on cattle and not humans. -The need to undertake tsetse control using the best methods have been identified but with no financial resources to support the plan. -Tsetse-infested forests that have been cleared for cotton growing have disrupted tsetse habitats due to chemicals used. -Current conservation strategies have sustained the preservation of tsetse flies and African trypanosomiasis.	1. Need for cross-sector, interdisciplinary decision making to stop rival narratives leading to competing actions. 2. Need for a One Health approach to break down the barriers between social scientists, natural scientists and the expertise of the community.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Mweempwa et al., 2015) [41]	To establish the impact of habitat fragmentation on the physiological and demographic parameters of tsetse flies in order to enhance the understanding of the relationship between fragmentation and AAT risk	Longitudinal study of tsetse age, abundance and trypanosome infection in areas of varying degrees of habitat fragmentation in eastern Zambia.	-A set of 3200 *Glossina morsitans morsitans* were caught using black screen fly rounds. -Overall, 577 female tsetse flies were dissected for ovarian age estimation. -A sentinel herd of 40 cattle was established at each of the four sites of Katete and Mambwe districts.	-Results indicated a significant increase in tsetse age as fragmentation increased. -Tsetse density was lower in most fragmented areas whilst the proportion of female flies increased significantly as fragmentation reduced. -AAT incidence in cattle was determined using buffy coat method. Infection rate in both cattle and tsetse flies was higher in highly fragmented areas.	1. Need to develop models that link biological characteristics of tsetse flies with habitat conditions. Such models maybe helpful in planning tsetse control interventions.
(Meyer et al., 2016) [25]	A literature review of past and on-going tsetse and African trypanosomiasis programmes	Systematic literature review of tsetse and African trypanosomiasis programmes between 1980 and 2015	Five African countries including Zambia. 68 documents plus 12 structured questionnaires reviewed.	-Twenty-three major Tsetse and Trypanosomiasis control programmes recorded from the five countries. Three control programmes conducted in Zambia during the stated period include the following: - Insecticide treated targets and traps (ITT) + trypanocidal drugs (TRY) in western province under government services for tsetse elimination (1987–1989). - Sequential aerial spraying (SAS) + ITT in eastern province under Regional Tsetse and Trypanosomiasis Control Programme (RTTCP) for tsetse control (1989–1994) -SAS + ITT in Kwando Zambezi belt under Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) for tsetse elimination (2008 onwards)	1. Need for evaluation of the control programmes recorded. 2. Need for standardised protocols to conduct such evaluations of control programmes
(Alderton et al., 2016) [15]	To develop an agent-based model (ABM) for investigating *Trypanosoma brucei rhodesiense*	-Mixed methods	-ABM comprised of human/animal trypanosomiasis and tsetse ecological survey data obtained along the 75km transect in the Luangwa valley of Zambia. -Ethnicity, age and gender data were also incorporated.	-ABM produced output that could not be readily generated by other techniques. On average there were 1.99 (S.E. 0.245) human infections and 1.83 (S.E. 0.183) cattle infections per 6-month period. -The model output identified that the approximate incidence rate (per 1000 person-years) was lower amongst cattle owning households (0.079, S.E. 0.017), than those without cattle (0.134, S.E. 0.017). - Immigrant tribes (e.g., Bemba I.R. = 0.353, S.E.0.155) and school-age children (e.g., 5–10-year-old I.R. = 0.239, S.E. 0.041) were the most at-risk for acquiring infection.	1. The ABM can be used as a tool for scenario testing at an appropriate spatial scale to allow the design of logistically feasible mitigation strategies suggested by model output. This is of importance where resources are limited, and management strategies are often pushed to the local scale.

Table A1. *Cont.*

Author Year	Study Aim	Study Design	Sample and Participation	Study Findings	Needs Domain
(Holt et al., 2016) [2]	To assess AAT vulnerability in cattle owing communities	Cross sectional survey of cattle owners using questionnaire interviews.	210 households from Lundazi and Mambwe districts of Zambia	-AAT was constant with seasonal pattern, some trypano-tolerant breeds and communal grazing, small/moderate herd size with crops and mixing farming as primary income source, losses to draft reported, slightly higher mortalities and moderate costs diagnosing and treating, less likely to report treatment failure, low/good knowledge of control and tsetse traps/targets reported. -moderate AAT challenge, some concerns with resistance reported and most likely to keep pigs while some keep sheep and goats.	1. Need to integrate novel treatments with new and existing diagnostic and control programmes with findings of the study to develop tailored recommendations for AAT control and the reduce its impact in vulnerable communities.
(Meyer et al., 2018) [27]	To propose a framework for conducting a cost benefit analysis of possible AAT control analysis	A literature review of AAT of cattle production, herd management, impact of AAT on productivity, incidence and mortality	Two districts from Cameroon and Zambia (Mambwe district)	-For Zambia, the 10-year impact of tsetse elimination on the net value of cattle production was calculated as benefit–cost ratios using a discount rate of 5% and indicated the following:-2.3 (1.8–2.7) Targets, insecticide treated cattle (ITC) barrier -2.0 (1.6–2.4) Targets, barrier traps -2.8 (2.3–3.3) Aerial spraying, ITC barrier -2.5 (2.0–2.9) Aerial spraying, barrier trap -The use of SAT as elimination method for Mambwe district yielded a higher benefit–cost ratio than the use of targets. -The model estimated the total discounted control costs at 3.8 million USD and benefits at 10.5 million USD for Mambwe district if SAT was used as tsetse elimination method	1. Need for barriers to be maintained and monitoring activities conducted continuously unless sequential elimination of the entire tsetse belt is achieved. 2. Cost–benefit studies should be supported by recent estimates of key parameters such as frequency of trypanosome infection and impact, livestock and tsetse demographics. 3. Model generated in study combined data from different locations and from studies conducted years ago, there is need to validate the model using current data from same locations. 4. Need to use existing control programmes for designing future control programmes.

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Tropical Medicine and Infectious Disease

MDPI

Opinion

Assessing Climate Change Impact on Ecosystems and Infectious Disease: Important Roles for Genomic Sequencing and a One Health Perspective

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Abstract: Changes in the Earth's climate and weather continue to impact the planet's ecosystems, including the interface of infectious disease agents with their hosts and vectors. Environmental disasters, natural and human-made activities raise risk factors that indirectly facilitate infectious disease outbreaks. Subsequently, changes in habitat, displaced populations, and environmental stresses that affect the survival of species are amplified over time. The recurrence and spread of vector-borne (e.g., mosquito, tick, aphid) human, animal, and plant pathogens to new geographic locations are also influenced by climate change. The distribution and range of humans, agricultural animals and plants, wildlife and native plants, as well as vectors, parasites, and microbes that cause neglected diseases of the tropics as well as other global regions are also impacted. In addition, genomic sequencing can now be applied to detect signatures of infectious pathogens as they move into new regions. Molecular detection assays complement metagenomic sequencing to help us understand the microbial community found within the microbiomes of hosts and vectors, and help us uncover mechanistic relationships between climate variability and pathogen transmission. Our understanding of, and responses to, such complex dynamics and their impacts can be enhanced through effective, multi-sectoral One Health engagement coupled with applications of both traditional and novel technologies. Concerted efforts are needed to further harness and leverage technology that can identify and track these impacts of climate changes in order to mitigate and adapt to their effects.

Keywords: biosecurity; climate change impact; One Health; genome; sequencing; infectious disease

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> *Climate change impacts transcend international borders and geographic areas of responsibility.*
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> CNA Military Advisory Board, National Security and the Accelerating Risks of Climate Change.

1. Introduction

As part of a panel discussion at the 2019 Sequencing, Finishing, and Analysis for the Future (SFAF) conference (21–23 May, 2019, Santa Fe, New Mexico, USA), we explored the importance of using genomic sequencing in a One Health context to study climate change impacts on natural and managed ecosystems and their infectious diseases (Figure 1). We summarize key discussion topics in which the application of genomic sequencing technologies can enhance our understanding of climate

change and infectious diseases using a One Health approach. The topics included an array of scientific applications including current sequencing tools, genomics and environment, science of signatures and conservation genomics, plant ecosystems, a research case study, and outcomes of human behavior such as data and material sharing. The importance of integrating these applications across One Health, a multi-disciplinary, and multi-sectoral approach helps demonstrate how the entire work can be related and interact. Recent effects of relevant infectious diseases linking our discussion to the importance of neglected tropical diseases are also addressed.

Unprecedented opportunities for studying microbial populations have been created by current sequencing technologies [1]. For pathogens with comparatively low per-site mutation rates, such as DNA viruses and bacteria, Biek et al. [1] point out at that whole-genome sequencing can reveal the accumulation of novel genetic variation between population samples taken at different times. The concept of "measurably evolving populations" and related analytical approaches have provided powerful insights for fast-evolving RNA viruses, and sequencing technologies are now economical enough to apply to other DNA microbial pathogens [1]. These methodologies provide unprecedented windows to understanding how pathogens are adapting or evolving in real time in the face of climate change.

Climate change due to higher global temperatures and changing precipitation patterns affects the environment, altering coastlines and exacerbating flooding, and contributes to extreme weather patterns, all of which are influenced by the type, magnitude, and rate of change [2]. Here, climate change impact is defined as those effects resulting from climate change drivers such as deviations from average temperature and precipitation over several decades of time. For example, human, animal and plant systems are affected by rising atmospheric (greenhouse) gases coupled with globalization and other influences including cultural, economic, political, and technological factors [3,4]. Climate change is already affecting populations and infectious disease dynamics among wildlife, plants, insects and other living things, altering host and disease vector life cycles, shifting vector, pathogen and host ranges as well as migratory patterns, along with disease spread and its attributable impacts. The fact that neither transboundary infectious disease agents nor climate impacts respect international borders [5,6] reinforces the need for regional and global approaches that encourage open communication and transparency.

There are multiple scales of impact of changing climatic conditions—the habitats and plants that are responding to a changing climate, the vertebrate organisms that are adapting to the changing habitats and climate, and the microbes that are evolving to the new conditions at each level. The core thread between each of these biological scales is the genome. Genomes in all organisms evolve and adapt to new conditions over time. The genome may change by gene expressions in response to the environment or there may be modifications in the genetic code itself. Following the changes in the genomes of organisms at the multiple scales in response to changing conditions requires the measurement of the genome, which is primarily accomplished through next generation sequencing. In the One Health construct of considering the health of humans, animals, and the environment together, the genome is a foundational element for measuring the adaptation these systems over time.

Environmental changes caused by natural disasters and human-made accidents raise risk factors that indirectly increase infectious human disease outbreaks as a result of displaced human populations, constrained resources, and damaged infrastructure [7]. When variables including health, socio-economic, and political factors are considered together, climate change and its impacts can be thought of as "threat multipliers" [8,9]. Addressing these challenges through a holistic One Health approach recognizes and integrates perspectives related to animals, humans, and environment [8]. Key to the environmental aspect of the One Health concept is the importance of plant systems, which provide food and fiber and support the economy, contributing to socio-economic and political stability [3,10]. Both agricultural and environmental change are inextricably linked to climate change and, like higher vertebrate species, microbial pathogens will either adapt or not.

The design of effective programs for scientific inquiry, modeling, response to and mitigation of climate change impacts on biological systems will require a multi-sectoral, cross-disciplinary approach. For example, plant systems are often omitted from One Health considerations despite their direct nutritional and ecological relationships to animals and humans. Here, we note that plants and their phytobiomes have critical roles in both food security and climate change. One consequence is that the loss of agricultural productivity resulting from climate change can impact food security directly (reduced food production) or indirectly (the impact of rising global temperatures on food access, utilization, and safety) [11]. Furthermore, there is a link between food security, social stability and national security [3], as inadequate food supplies may trigger social unrest and/or human movement and migration. The impacts of these changes may be most severe among populations that are less affluent and those in the tropics [3,11,12]. Addressing these issues must involve key players from the climate, environmental, and genomics sciences and members of the security sector. In addition, public-private partnerships will help to galvanize national government level activity with those of industry and civil society.

2. Current State of Sequencing Tools

Tools and associated methods available for One Health-based responses to climate change impacts include both gold standard quantitative real-time PCR (qPCR) and current metagenomics sequencing approaches, which are less biased [13]. Their application can provide increased ability to detect and identify emerging and new pathogens and biological invasion by invasive species that are expected to increase due to climate impact [14,15]. Recently, the newly emerged coronavirus, which was first called the 2019-novel coronavirus (2019-nCoV) and now designated as the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was first reported in four cases associated with a wholesale market in Wuhan, China on 29 December 2019; [16]. The speed and transparency of the early work to understand this newly emerged coronavirus is remarkable; the full genome was released to the world on 10 January 2020. The release of the SARS-CoV-2 genome led to an almost real-time understanding of the viral phylogenetics, and the deduction that it came from a wildlife wet market, jumping from animal species [17] commonly bought at the market for consumption. With human populations, wildlife reservoirs, vectors, and microbes adapting in response to rapidly changing environmental conditions, outbreaks of pandemic potential such as that caused by SARS-CoV-2, will occur more regularly.

Genomic sequencing can be applied across many disciplines which are also relevant to climate change impacts, One Health, and biosecurity. A chart detailing a framework of how a variety of genomic tools can be applied to better understand various aspects of biological invasions [15,17] can help to facilitate outbreak response, enhance understanding of the role of changing environment and facilitate management of priorities through each stage of the invasion process. This framework of tools is also relevant to tracking certain neglected tropical diseases such as schistosomiasis, which is caused by parasitic flatworms and transmitted by exposure to contaminated water sources. Freshwater snails serve as the intermediate host of the flatworms. Climate change is expected to continue drier and hotter conditions across many parts of Africa, which should decrease the incidence of schistosomiasis by reducing the extent and availability of snail habitats [18]. Genomic sequencing can be applied here to track and multiple organisms and detect the presence of various species within a given habitat.

Developing high-quality pathogen detection methods used in clinical diagnostics, infectious disease surveillance, and forensics requires an understanding of the true genetic diversity of the pathogens, their ecology, geographic distribution, reservoirs, hosts, vectors, and genetically related organisms. Using a priori approaches to design molecular detection assays can lead to signature erosion. In the H1N1 influenza pandemic of 2009, for example, a genetic shift in the pathogen resulted in the inability of existing PCR assays to detect that strain [19]. The more we look at the genetic diversity of a particular pathogen, the more we discover, including unique sequences that reduce the sensitivity or specificity of existing diagnostics or detection methods.

Pathogen genetic diversity can be a hindrance to assay development as in the case of Lassa fever, which has symptoms similar to malaria and viral hemorrhagic fevers such as Ebola, and related co-infections among patients. Due to the complex diversity of the Old World arenavirus causing Lassa fever, PCR assays specifc for detection of this virus are difficult to design despite their single transmission mode via contact with rodent reservoirs [20]. This is due to the large genetic diversity of viral sequences in the reservoir population. Each cluster of human disease outbreak appears to derive from a single rodent-to-human transmission event that randomly samples a small portion of the true viral genetic diversity. Therefore qPCR assays are often designed using a snapshot of the viral genetic diversity known available at the time. Subsequent disease outbreaks may reveal new genetic diversity, eroding the performance of existing assays. A comprehensive surveillance of the rodent reservoir would be needed to understand the true level of genetic diversity that assays should be designed to detect. The need for proper biosafety controls from secure sample transport to laboratory biocontainment especially in low resource areas reinforce the need for effective, timely, and low-cost diagnostics [21].

3. Genomics and a Changing Environment

Although both climate change impacts and the research needed to understand and mitigate them are long term, time is no longer on our side and our approaches to solving such complex problems will require creativity and expediency. In epidemiological or climatological modeling, for example, data collection is traditionally completed first, followed by model development. However, a recent trend is for the two to be implemented concurrently so that data gaps are recognized immediately and data collection can target those gaps in real time. Estimating population spread and impacts of scenarios for changing environments or newly emerging viruses is now possible given advances in ecologic and epidemiologic models [22,23]. Genetic research and sequencing analyses have shown correlations suggesting that invasive species (weeds) moving into agricultural landscapes require less flexibility and less genetic diversity than those moving within the natural environment [15]. Tracking plant movements can help to inform the development of tools for predicting future plant invasions and migrations, which are expected to increase as climate change-induced habitat disturbances become more widespread [15]. Such approaches increase our ability to predict shifts and trends, whether in climate changes or pathogen spillover.

With regard to sequencing technology and meeting the challenge of climate change, there are two primary ways that genomics can be applied immediately. The first, as mentioned above, is understanding host, vector and pathogen range shifts due to environmental change. The second is incorporating sequencing of archived sample sets to investigate how pathogens are both evolving and shifting into new hosts and ranges. Recent evidence finds climate change is already affecting wildlife populations and disease dynamics [24–27], altering host and disease vector life cycles as in case of certain NTDs, shifting vector and host ranges and migratory patterns [26,28], and changing disease spread and its impacts. The consequences of these climatically induced changes are poorly understood, generating the need for comprehensive research involving both genomics [27] and predictive modeling of infectious disease and species distributions [29]. A multitude of factors may influence how both hosts and vectors of infectious diseases respond to changing climates. Finding signatures of environmental change in biological communities, as well as fusing and analyzing multi-scale data across species, time, and latitude, will be key.

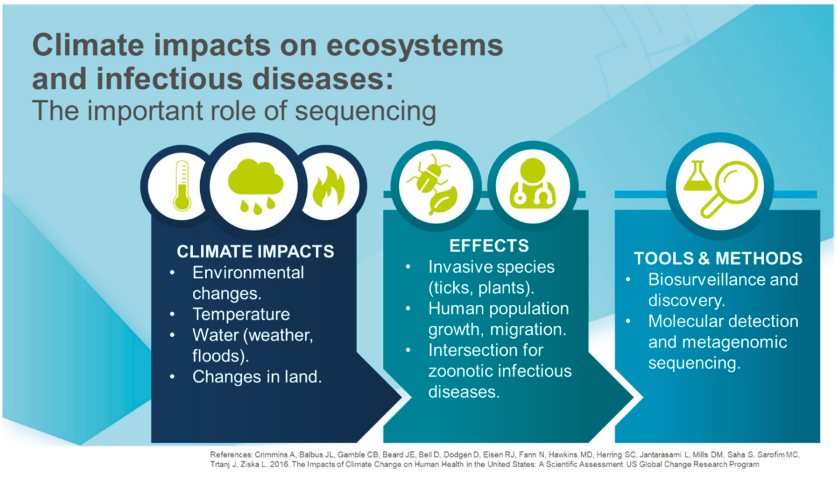

Figure 1. Climate impacts [30] whose effects on ecosystems and infectious diseases can be investigated using a variety of tools.

Combining sequencing and modeling will allow us to better understand how climate may be affecting geographic ranges of hosts, human migration, vectors, and parasites. The following questions can be asked using newly collected or archived samples from freezers around the world: How have pathogens in vectors and hosts changed over the last several decades? How do pathogens differ, at the genomic scale as well as in prevalence, geographically in both the hosts and vectors? Answering these sorts of questions is possible by simultaneously using sequencing with epidemiological models to make predictions that incorporate characteristics of pathogens, populations, and environmental variables.

4. Science of Signatures and Conservation Genomics

In order to understand the natural world, we must first find patterns that are otherwise invisible or indistinguishable from the chaos of natural systems. Finding such patterns can reveal signatures of long-term or short-term impacts of environmental events, evolutionary processes, or other idicators of environmental health. Using genomic sequences and bioinformatics to find and measure signatures in organisms can contribute to enhanced understanding of these complex systems and to better prediction of future impacts. In understanding the impacts of climate change on species, scientists are working to understand and predict which species will be able to adapt locally and which species will not be able to adapt [31,32]. Many of these adaptations will be through genetic adaptation to a changing environment [33].

Both marine and terrestrial ecosystems are under pressure, not only from climate change, but from over-exploitation, habitat loss and degradation, growing human populations, and ultimately species imperilment. Global climate change is altering our ecosystems at an unprecedented rate, threatening plants, animals, and the habitats in which they live. According to five coordinated reports in 2019 from the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), sponsored by the United Nations, over 1 million species are threatened with extinction [34]. Conservation genomics, which is closely linked to understanding the health of individuals and populations, can inform our efforts to manage infectious diseases and animal and plant health under these new environmental challenges. Understanding how ecosystems, and the populations within them, are likely to respond under different climate scenarios is essential for planning future conservation strategies and mitigations for infectious diseases. Modernizing our approach to natural resource management will ensure that we are utilizing the best available science to help make complex and difficult management decisions, and embracing sequencing where appropriate to find those signatures

of the health of populations will be as important as the application of sequencing towards infectious disease diagnosis and detection.

5. Plant Ecosystems

In both natural and agro-ecosystems, plants play critical ecological roles by utilizing carbon dioxide and releasing oxygen, emitting substrates into the soil and atmosphere, modifying soil moisture, and reducing the amount of rainfall reaching the ground [35]. Effective assessment of climate issues related to plants should consider the plant as a complex "phytobiome" composed of communities of species within an environmental niche [36], and aspects of climate change, particularly altered temperatures and rainfall, modify community diversity [3]. Crop plants are cultivated in disturbed ecosystems and, at least in developed nations, are typically planted in monocultures, adding to their vulnerability to attack by new species of invasive pests or pathogens.

Since infectious disease develops only in a conducive environment [37], climate change will be a key driver of changes in plant disease epidemiology. For example, even a small increase in the length of a growing season can significantly increase the amount of pathogen inoculum produced [38]. Some genes encoding host plant disease resistance factors are less effective at higher temperatures, and higher cool-season temperatures may favor the survival of insect vectors of some plant pathogens [38]. Changes in climate (e.g., higher temperatures, lack of water, increased carbon dioxide, and other environmental changes) impact each of these elements in different ways including environmental hardiness, changes in developmental rates and flowering times, productivity, shifts in geographic ranges, and virulence (for pathogens and other microbes) or disease susceptibility (for plants) [15,35].

6. Research Example and Case Study of One Health Approaches to Using Sequencing to Understand and Mitigate Climate Change Impacts

Sequencing can provide a unique opportunity to derive new information from archived sample sets, especially when historical and recent samples are compared directly. Investigation can focus on genomic changes in individual microbes or microbiomes, or from host organisms. Targeted approaches can select specific pathogens and identify how they are associated with environmental change.

Vibrio cholerae is a well-known model for studying water-borne diseases and as part of an ecosystem, combined with environmental dynamics has been termed as biocomplexity [39]. When combined with genomic sequencing, epidemiology, and remote sensing, non-cholera vibrios such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* represent a microbial barometer for climate change [40]. In 2016–2017, genomic sequencing showed that isolates in Yemen were *V. cholerae* serotype Ogawa isolates from a single sublineage of O1 El Tor [41]. This outbreak in Yemen which resulted in over one million cases and over 2000 deaths exemplifies the intersection of climate change, human disease, and related social factors of conflict and migration.

Zoonotic dieases such as plague and hantavirus caused by *Yersinia pestis* bacterium and Sin Nombre virus, respectively, are endemic in the US Four Corners region (Utah, Colorado, Arizona, and New Mexico). While the transmission of plague and hantavirus are different, the incidence of these viruses depends on rodent reservoirs and vectors (e.g., fleas). The occurrence of heavy precipitation in a given year often results in a larger rodent population, which increases risk of hantavirus transmission through interaction with humans. Models for predicting zoonotic diseases include diverse reservoirs of rodents in Central Asia, the Middle East, and the US Midwest which is adjacent to the Four Corners region [42]. Overall, the ability to collect and reconcile historical data with predictive modeling will be very powerful to understand transmission dynamics.

Case-Study: Expanding New Sequencing Technology Across the Globe to Detect Animal to Human Disease Spillover

Climate and health are intertwined with the effects of climate shifts that are currently manifesting globally. New technology, specifically in sequencing and metagenomics, must be implemented in geographically diverse regions around the globe in order to maximize impact. These newly developed or newly implemented technologies have the capacity to benefit even remote populations, who are often the most impacted by these health shifts. In addition to the identification of new pathogens, training on use of these technologies will have long-term impact by building local capacity to detect and respond to emerging health threats on a routine basis.

The USAID-funded PREDICT program [43,44] was implemented in over 30 countries around the globe from 2009–2020, aimed to strengthen capacity for surveillance and detection of viral threats transmitted from animals to humans. In order to address challenges of building and sustaining laboratory viral detection capacity in low-resource partner countries, the PREDICT approach built laboratory platforms based on conventional PCR methods to identify potential pathogens in wildlife and humans. Building upon this approach, the project also integrated more complex sequencing technology to empower local partner universities and governments to increase their viral detection capacity at the local level. Partner countries who successfully adopted this approach identified over 1000 new viruses at the human-animal interface. Recognition of the significant scientific and economic benefits of discovering high-consequence pathogens before evidence of spill-over into human populations, participating countries are now starting to realize the power and importance of sequencing and prioritize this technology as a key component to future capacity building [45]. This new focus in low-resource, viral hot-spot regions of the world promotes the potential for additional high-level metagenomic skills to be added to the cadre of possibilities. A critical piece of continuing the work of PREDICT is to encourage countries to store sample repositories for the long-term so that further work can be done utilizing mores powerful metagenomic approaches as they become available. As these countries are particularly impacted by drivers such as climate shifts, human and animal movement, and population growth, we anticipate continuing viral spillover at the human-animal interface, making the need for a continued increase and investment in pathogen detection capacity [46].

The sequencing technologies discussed in this paper, which expand our ability to detect pathogens of pandemic potential, should be deployed locally and globally in areas where spillover and disease spread continue to occur. The challenge is to continue a global dialogue to encourage development of affordable platforms and training that can lead to discovery of known and novel viruses with pandemic potential [47]. The One Health Institute at the University of California, Davis, led the PREDICT Project and is also home of the University of California Global Health Institute's Planetary Health Center of Expertise that works to raise awareness about climate and health issues as well as sustainable solutions and platforms (https://ohi.vetmed.ucdavis.edu). With the help of such centers, the global population at large is beginning to appreciate the importance of understanding local predictions for environmental change and disease spillover to utilize adaptation and mitigation measures. It has become well known that understanding drivers of pathogen spillover and risk, and utilizing state-of-the art technology for management of these threats, are critical. The authors are optimistic that there is a true space for development of easily accessible technologies that are farm, clinic or animal/human-specific, resulting in rapid, real-time on-site diagnostic care utilizing the new sequencing technologies discussed herein.

7. Importance of Trust and Sharing of Data and Material

There is a continued need to earn and build trust between partners at the working level of research groups in universities, companies and institutes, and the top level of government partners especially in host countries where work is being implemented. It is critical to invest time to build a collaborative network that respectfully supports trust through transparency and partnership. The ability to collaborate professionally with various partners is critical to generate and provide the various forms of data and technology (from field to lab), including the models and sequencing methods discussed herein. More frequently, funding and donor agencies are requiring data be made available in a public and open source manner. Occasionally frameworks for data sharing are provided, but other times the research team needs to develop an approach that works best to meet the requirements and needs. The ability to work in a global society beyond any individual research lab, or any individual

organization to generate a solution by working together should be recognized. One key to success is to have strong partnerships on the ground with protocols for data sharing in place from the beginning.

8. Conclusions

Through our discussion, we draw attention to the power of genomic sequencing that complements expertise and tools across disciplines and sectors to address climate change impacts on ecosystems and infectious diseases. Using a One Health approach is important to view the problem on a larger scale, and beyond the scientific and technical work the government policies developed in some countries are now starting to take a coordinated One Health multi-sectoral approach to implement change. We described some examples of climate change impacts on animal and human infectious disease that exist in vector-borne, zoonotic, and neglected tropical diseases. Climate change is also a key driver of multiple changes in plant epidemiology, affecting disease susceptibility and resistance as well as environmental hardiness, developmental and flowering rates, productivity, and geographical range. Genetic tools facilitate the prediction of such changes and help in the development of management options. Shaping human behavior is key to positive and practical outcomes. Besides the importance of shared goals and trust-building as related to setting research agendas and sharing intellectual resources, another critical theme that arose is the need to increase partnering, especially for engaging public and private partners that can help incorporate a multi-sectoral approach with a diverse audience of stakeholders who will form policy. This audience goes beyond public and animal health specialists and targets traditional biotechnology as well as social and life scientists that have expertise in behavior change, food safety and security, the environment, hydrology, climate, and meterology. The practice and policies developed will shape funding which, in turn, will leverage the commercial partnerships that are challenged with pursuing markets with varying returns on investments and are also critical in sustaining programs through the economy and enterprise. This combination of resources and expertise contributes to a more open and collaborative approach to understanding the links between infectious disease and climate.

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Tropical Medicine and Infectious Disease

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Article

Lessons Learned and Paths Forward for Rabies Dog Vaccination in Madagascar: A Case Study of Pilot Vaccination Campaigns in Moramanga District

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Abstract: Canine rabies causes an estimated 60,000 human deaths per year, but these deaths are preventable through post-exposure prophylaxis of people and vaccination of domestic dogs. Dog vaccination campaigns targeting 70% of the population are effective at interrupting transmission. Here, we report on lessons learned during pilot dog vaccination campaigns in the Moramanga District of Madagascar. We compare two different vaccination strategies: a volunteer-driven effort to vaccinate dogs in two communes using static point vaccination and continuous vaccination as part of routine veterinary services. We used dog age data from the campaigns to estimate key demographic parameters and to simulate different vaccination strategies. Overall, we found that dog vaccination was feasible and that most dogs were accessible to vaccination. The static-point campaign achieved higher coverage but required more resources and had a limited geographic scope compared to the continuous delivery campaign. Our modeling results suggest that targeting puppies through community-based vaccination efforts could improve coverage. We found that mass dog vaccination is feasible and can achieve high coverage in Madagascar; however, context-specific strategies and an investment in dog vaccination as a public good will be required to move the country towards elimination.

Keywords: canine rabies; mass dog vaccination; central point vaccination; puppy vaccination; Zeroby30

1. Introduction

Canine rabies results in an estimated 60,000 human deaths per year globally [1]. These deaths are entirely preventable: prompt post-exposure prophylaxis of humans exposed to rabies is highly effective at preventing death and mass dog vaccination can interrupt transmission in domestic dogs and eventually lead to disease elimination [2]. The World Health Organization (WHO) and its partners have set a goal to eliminate human deaths due to canine rabies by the year 2030 ("ZeroBy30") [3]. Annual dog vaccination campaigns achieving at least 70% coverage are the recommended target for controlling rabies in domestic dog populations [4]. However, achieving this coverage target in low- and middle-income countries where the burden of human rabies is highest can be challenging due to economic, ecological, sociocultural, and political barriers [5].

In sub-Saharan African countries, parenteral vaccinations implemented through static point campaigns have been shown to be cost-effective and feasible [6]. While most dogs in these settings are considered free roaming, they are mostly owned and are accessible for vaccination through these campaigns [7,8]. However, reaching high coverage and maintaining vaccination campaigns at that scale requires sustained investment and coordination, and the challenges in implementation largely reflect financial and logistical constraints more than the feasibility of vaccination itself [5]. Developing clear and context-specific strategies and lowering costs and resources needed could help spur the implementation and scaling up of campaigns in these countries.

In Madagascar, canine rabies has been an endemic for over a century, and for most of that period, the Institut Pasteur de Madagascar has provided post-exposure prophylaxis free-of-charge to bite patients in the country [9]. Currently, there are only 31 clinics where these human vaccines are available, with one clinic serving on average greater than 700,000 persons and over 20,000 bite patients treated annually [10]. There is minimal dog vaccination due to high costs to owners and a lack of vaccine availability [11]. Recent studies have estimated a high burden of human rabies deaths (approximately 1000 deaths annually), masked by weak surveillance across the country [10,11].

The veterinary sector is largely private and practices are largely limited to urban areas, but approximately 204 veterinarians are employed in hybrid private/public employment as designated district veterinarians by the national government. While dog vaccination is rare, livestock officers and veterinarians work together to implement cattle vaccination campaigns for anthrax on an annual basis as mandated by the government (owners are charged a fee per animal for these vaccines, which vary by location) [12]. No routine mass dog vaccinations have been conducted on the island, although a few pilot programs have begun in recent years, largely implemented by NGO–government partnerships.

Here, we summarize lessons learned through the implementation of pilot vaccination programs in the Moramanga District of Madagascar, where previous work has shown high incidence of dog rabies cases and human rabies exposures. In 2018 and 2019, we deployed two different vaccination strategies. In 2018, we carried out a larger scale volunteer-led pilot vaccination campaign in two communes (sub-district level) using a static point strategy where owners brought their animals to a fixed location for vaccination. In 2019/2020, we provided vaccines, all necessary supplies for vaccine administration, and a per-vaccine fee to the district veterinarian to vaccinate animals as part of a continuous vaccination strategy, where vaccines were delivered alongside routine services provided by the veterinarian. We compare the time, human resources, costs, and coverage estimates between these campaigns, and using a demographic and vaccination model, we further explore different vaccination strategies based on what we learned during implementation.

2. Methods

2.1. Study Area

The Moramanga District is located midway between the central highlands and the east coast of Madagascar at an average altitude of 936 m. It comprises 21 communes, covering approximately 7150 km^2 with an approximate human population of 347,000 [13]. Previous work in the district has established a high burden of rabies exposures (42–110 per 100,000 persons) and deaths (1–3 deaths per 100,000 persons) despite the availability of post-exposure prophylaxis at the district hospital [11]. While Moramanga is relatively close to the capital city of Antananarivo (~3 h by bus), within the district, travel times between locations are highly variable, with much of the population living in more rural areas with limited access to roads and transportation [10]. Before 2018, there were limited animal rabies vaccination services, with most animal vaccines available in the urban commune of Moramanga Ville, where owners were often charged > 15,000 Ariary (~4.28 USD) per vaccine administered.

2.2. 2018 Campaign

In 2018, two NGOs (the Mad Dog Initiative (MDI) and Traveling Animal Doctors (TAD)) organized a pilot vaccination campaign in collaboration with the Department of Veterinary Services and the Ministry of Public Health in the District of Moramanga Figure 1. This campaign focused on two communes in Moramanga, Moramanga Ville (the district center) and Andasibe (a rural commune surrounding Andasibe National Park), where previously high incidence of probable rabies exposures (Moramanga Ville) and a high burden of deaths (Andasibe) had been recorded [11].

The campaign was planned as a series of static point vaccination stations covering 1–3 fokontany (i.e., sub-communes) per day (see Figure 1). A week before the campaign dates, the vaccination team informed the chief of the fokontany about the campaign and provided fliers advertising the date of the vaccine and that it would be available at no cost to owners (Figure 1). During the campaign, we used Rabisin (10 mL vials with 1 mL per dose, Boehringer Ingelheim) to vaccinate both dogs and cats presented that were at least 1 month old based on current WHO recommendations for endemic settings [4,14]. Rabisin has a manufacturer-stated duration of protection of 1 year given one dose and of an additional three years if an additional dose is given approximately one year after the first dose. As part of the campaign, owners were surveyed by vaccinators about how many dogs and cats they owned in total (split by >1 year vs. <1 year in order to avoid language ambiguities that might result in excluding puppies and kittens) as well as if their dogs were free roaming (no restrictions on movement by the owner/'mirenyreny', tied/'mifatora', or fenced/'mifefy'). Vaccinations were delivered at no cost to owners, but as animal vaccinations are generally thought of as a paid service in Madagascar, owners were asked how much in Ariary they would be willing to pay to have one animal vaccinated against rabies (after being informed that the current vaccination was free). For each animal vaccinated, we recorded the species (cat or dog), sex, approximate age in years as reported by the owner, and whether the animal had been previously vaccinated.

To assess coverage, post-vaccination coverage surveys were conducted according to a previously established methodology [15,16]. All animals vaccinated were concurrently marked with a colored, nontoxic, livestock crayon along the top of the head or back. At the end of each campaign day between 1600–1800 h, when dogs are most active [15], two transect surveys were conducted on vaccination campaign days in each vaccination location by two teams (consisting of two volunteers and one local guide) for one hour on separate paths and in opposite directions. Marked and unmarked dogs were recorded as well as their roaming status (i.e., roaming, inside a fence, or tied), and their approximate age (>1 year or <1 year of age).

Figure 1. Photos from the 2018 campaign. **Top left**: advertisement for the campaign posted on the door of the fokontany office as the campaign starts; **top right**: a dog post-vaccination marked with a crayon; **bottom left**: a basket of puppies brought for vaccination; and **bottom right**: a line of owners and dogs waiting for vaccination. Photo credit: Jochem Lastdrager, Traveling Animal Doctors.

2.3. 2019 Campaign

For the 2019 campaign, instead of a static point campaign strategy, vaccine vials (Rabisin) and the supplies needed to administer them (needles, syringes, and vaccination cards for owners) were distributed to the district veterinarian, who then delivered the vaccination at no cost to owners but was directly compensated 1500 Ar (~0.40 USD) per rabies vaccine administered. The campaign lasted from 6 September 2019 to 19 June 2020. One week prior to her visiting each location, the district veterinarian advertised the vaccines by calling ahead to the fokontany leaders and other officials who then advertised to their communities, largely through word-of-mouth. For each animal vaccinated, the district veterinarian collected the animal's age and sex and asked owners to approximate the distance they travelled to receive the vaccination in meters. Researchers communicated with the district veterinarian about progress periodically throughout the campaign, primarily through telephone calls. No other compensation or instructions were provided, and we asked the district veterinarian to administer as many (or as few) as feasible or wanted. As the vaccinations were delivered continuously, we were unable to do comparable post-vaccination surveys.

2.4. Analyses

2.4.1. Campaign Resource and Cost Comparisons

We documented the overall costs and resources required for the two vaccination efforts. We tracked the number of vaccination points, the number of days over which these vaccinations occurred, and the number of person days required overall (i.e., the number of working people per day over the campaign [17]) in addition to monetary costs. As costs were incurred in both USD and Ariary and as the exchange rate declined rapidly between 2018 and 2019, we used the midpoint between the two years (3314 Ar to 1 USD) for the cost comparisons.

For the 2018 campaign, we broke costs down into the following categories: direct vaccine costs (cost for vaccine, syringes, needles, and vaccination cards), supplies (livestock crayons, muzzles, gloves, alcohol, and swabs), food and lodging for NGO personnel and other vaccinators during the campaign, personnel costs (per diems for the district veterinarian, livestock field officers, local guides, and NGO employees), and advertisement (posters and banners for advertising the campaign). Foreign NGO volunteer expenses for travel to Madagascar were not included in these costs. Vehicles and drivers were also not included in these costs, as the drivers' time and vehicle use were donated to the campaign by volunteers involved in the campaign. In 2019, costs were split into two categories, direct vaccine costs (for the same items as in 2018) and personnel costs (per vaccine fee paid to the district veterinarian), and supplies (a generator and fuel for the veterinarian to maintain the vaccine under cold chain during power outages). Transportation costs were also not included as the district veterinarian used her own vehicle and vaccinated as part of their routine veterinary service provisioning.

We used the data on owners' reported willingness to pay for vaccines to estimate the proportional reduction in animals vaccinated as fees are increased. We also estimated how this would impact cost per animal vaccinated by approximating the costs for implementation (i.e., those costs that remain fixed) from costs incurred per animal vaccinated (i.e., vaccine, syringe, vaccination card, and per vaccination fee to the district veterinarian in 2019) and by calculating the balance between the returns from owner payments (i.e., increases in cost recovery per animal vaccinated) vs. decreasing numbers of animals vaccinated overall.

2.4.2. Coverage Estimates

For the 2018 campaign, we used the transect data to estimate vaccination coverage as the proportion of dogs sighted that were marked using a binomial confidence interval at the commune level. For the 2019 campaign, we estimated the vaccination coverage using human-to-dog ratios (HDRs) and human population estimates [18,19]. We used a ratio range of 8–25 humans-to-dogs, based on previous data from Madagascar [20] and based on recent estimates from household surveys in the Moramanga District [21]. We set the point estimate using an HDR of 19.5, the midpoint between the HDRs estimated for two communities in the district by LeBlanc et al. 2019. We used human population estimates from the 2018 national census in each commune where the vaccinations were delivered [13]. Coverage was estimated as the number of dogs vaccinated in total in that commune divided by the estimated dog population. We used this same method for the 2018 campaign as well to compare the coverage estimated by the post-vaccination transects vs. HDRs.

2.4.3. Dog Demography

Using the age data on vaccinated animals collected during both vaccination campaigns, we estimated the proportion of population in four age classes: puppies under the age of 1 year, juveniles aged 1–2 years, adults aged 2–6 years, and older dogs aged 6+ years based on broad patterns of survival in comparable dog populations (i.e., low survival in the first year of life, followed by plateauing survival probabilites [22,23]). With the assumption that these estimates represent the population at a stable age distribution, we used a Leslie matrix

model to estimate annual adult survival probability and fertility using maximum likelihood estimation [24]. Specifically, we assumed that the number of individuals in each age class follows a Poisson distribution, with the mean predicted by the stable age distribution from the model (the proportion of individuals in each age class at equilibrium, equal to the eigenvector associated with the dominant eigenvalue of the matrix ν) multiplied by the total number of individuals in the population (N_t):

$$N_a \sim Pois(\nu N_t)$$

We assumed that all individuals older than 1 year of age reproduce and we did not estimate declines in fertility given the small proportion of dogs older than age six years in the population. To obtain bootstrapped estimates, we used 100 subsampled data sets of 1000 observations each from the observed age data to fit the parameters and varied the initial values used in the optimization (N = 100 initial values sets) for 10,000 parameter estimates total.

2.4.4. Modeling Vaccination Campaign Strategies

We used the parameter estimates from the demographic model to simulate different vaccination strategies in a hypothetical commune with 1000 dogs. We used a discrete time age-structured model with a monthly time step to compare three strategies:

(1) annual vaccination campaigns occurring within the same month each year targeting dogs of all ages

(2) continuous vaccination of new puppies throughout the year targeting puppies that reach the age of 3 months

(3) a combined approach with annual campaigns (as 1) and routine puppy vaccination (as 2)

We split the dog population into puppies (<1 year old) and adults based on the stable age distribution estimated from the demographic model. To estimate pup survival in year one, we took the fertility estimates (i.e., number of new puppies per reproducing dog observed in the pup age class) and divided by an estimate of newborn pups per reproducing dog each year based on average litter size, average number of litters per female per year [7,22,25], and the proportion of the adult population that is female (estimated from our data). We assumed that, for the annual campaign strategy, surviving vaccinated adult dogs were revaccinated in subsequent years [26] but that, if a pup had been vaccinated within 9 months of the campaign, it was not revaccinated. We also assumed that vaccine immunity lasted for a discrete period of 3 years (with revaccination resetting immunity). A subset of parameter estimates resulted in estimates of population decline, but based on the shape of the age pyramid and to simulate reasonable campaign scenarios, we filtered to parameter estimates that corresponded to positive population growth.

2.5. Data and Ethics Statement

All data were analysed in R version 4.0.2 (2020-06-22) [27], largely using the `tidyverse` package suite [28]. Geospatial data were mapped using the sf [29] package. All data and code are archived at https://doi.org/10.5281/zenodo.4663084, accessed on 30 March 2021 and available at https://github.com/mrajeev08/mora_vax, accessed on 30 March 2021. The vaccinations were part of a public health campaign and routine veterinary service provisioning carried out by the local veterinary officials and the NGOs involved and in partnership with the Ministry of Public Health and the Department of Veterinary Services at the national level. MDI also maintained the national research permits (MICET permit: #130-19/MEDD/SG/DGEF/DGRNE) for its research and volunteer programs. Prior to vaccination, verbal informed consent was obtained from animal owners, and owners could opt out of answering any questions or services provided. No personally identifiable information was collected at any point during the campaigns.

3. Results

3.1. Summary of the 2018 and 2019 Campaigns

During the 2018 campaign, a total of 3137 animals were vaccinated (2057 dogs and 1080 cats) in the Moramanga (urban) and Andasibe (rural) communes over 13 days during the month of April (Table 1). We vaccinated at 7 points in Andasibe and 14 points in Moramanga Ville. During the 2019 campaign, between September 2019–June 2020, the district veterinarian vaccinated a total of 2385 animals (1898 dogs and 486 cats) over 48 days in seven communes in the Moramanga District. While more animals were vaccinated per vaccination point and per vaccination day in 2018 compared to 2019, the number of animals vaccinated per person-day was much higher for the 2019 campaign. More animals were vaccinated in 2018 vs. 2019, but this was largely a result of vaccinating more cats during the 2018 campaign (Table 1).

In 2018, 15% of dogs had a previous history of vaccination (largely in the urban commune of Moramanga Ville), with only 7% of dogs vaccinated within the last year. This remained largely the same in 2019 (~13%), as the district veterinarian focused their efforts in other communes. The district veterinarian did vaccinate 771 dogs in Moramanga Ville in 2019, and of those, 24.9% had been vaccinated in the previous year's campaign whereas only 4.9% of dogs vaccinated in all other communes had any history of previous vaccination. In addition, in 2019, 2.1% of animals had been spayed or neutered, reflecting efforts by the Mad Dog Initiative to implement free spay and neuter clinics in the district.

In 2018, 19% of owners reported that their animals were free-roaming, but this varied by location (Table 1). In addition, while less than 19% of owners in Moramanga Ville reported that their animals were free roaming, the majority of animals observed during the transects (77%) were observed outside of fences and not tied, and thus, the majority of animals could be classified as semi-confined in the more urban township of Moramanga Ville and free-roaming in the rural setting of Andasibe (approximately 67% of owners reported their dogs as free-roaming, Table 1). In 2019, the district veterinarian also asked owners to approximate how far they travelled in meters to get their animals vaccinated and 94% of people reportedly travelled less than 1 km to reach the vaccination point.

Table 1. Summary of the 2018 and 2019 campaigns. Breakdown of the animals vaccinated, prior vaccination history, dog demography, dog ownership, and daily and per vaccination rates by year and location (for 2018).

	2018			**2019**
	Andasibe	Moramanga Ville	All Communes	All Communes
Total animals vaccinated	528	2609	3137	2385
Total dogs vaccinated	254	1803	2057	1898
Dogs with history of vaccination	5%	16%	15%	13%
Dogs vaccinated within last year	5%	7%	7%	13%
Percent male dogs	55%	56%	56%	65%
Average dogs per owner	0.8	1.1	1.0	–
Percent of owners with free-roaming dogs	67%	19%	28%	–
Animals vaccinated per day (total days)	88 (6)	372.7 (7)	241.3 (13)	49.7 (48)
Animals vaccinated per vaccination point (total points)	75.4 (7)	186.4 (14)	149.4 (21)	37.3 (64)
Animals vaccinated per person day (total person days)	11.7 (45)	21.6 (121)	18.9 (166)	49.7 (48)

3.2. Cost Comparison and Willingness to Pay

The 2018 campaign cost more overall and per animal vaccinated than the 2019 campaign, largely due to increased personnel costs (Figure 2A,B and Table 1). Reflecting the extra personnel necessary to run the static point campaign, the 2018 campaign also took substantially more person-days per animal vaccinated (Table 1). We found that charging owners for vaccinations would result in minimal cost recovery (Figure 2C) and, beyond a minimal cost, would actually result in increased costs per vaccinated individual than free-of-charge campaigns. Cost recovery would be more likely given a 2019 style strategy, where the majority of the costs are incurred on a per animal basis, compared to the 2018

campaigns, where the costs were largely due to setting up the static point vaccination stations (Figure 2A,B).More importantly, in all cases, even a nominal fee would significantly reduce the numbers of dogs vaccinated and thus vaccination coverage, particularly in the rural commune of Andasibe (Figure 2D).

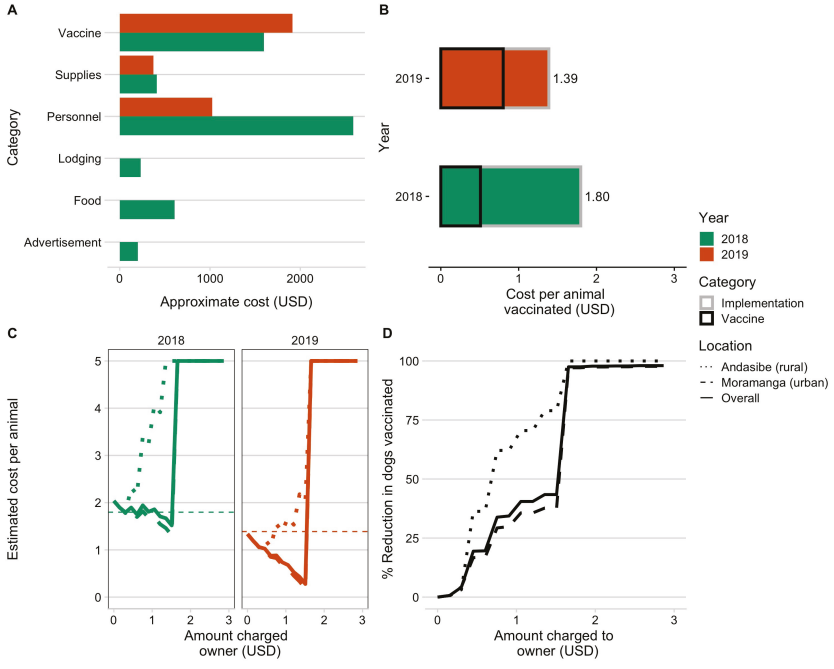

Figure 2. Comparing campaign costs and willingness to pay. (**A**) Vaccine costs broken down by category and by year (colors). (**B**) Overall cost per animal vaccinated for the two campaign years split by direct costs of vaccination per animal (i.e., vaccine, vaccination card, and syringes) and baseline implementation costs (i.e., personnel, supplies, subsistence costs for vaccinators during the campaign). (**C**) Estimated cost per animal vaccinated under a willingness-to-pay model for two campaigns examining increasing costs charged to the owner, with estimated costs declining due to cost recovery through owner payments and then peaking once owners reported no longer being willing to pay for the vaccine. (**D**) The percent reduction in number of animals vaccinated given owners' willingness to pay. The curves in C and D are shown based on the overall responses to willingness to pay (solid line) from both Moramanga and Andasibe, and the responses split by commune (dashed and dotted lines).

3.3. Comparing Campaign Coverage Estimates

The 2018 campaign covered two communes and was estimated to have achieved approximately 60% coverage (Figure 3A). The 2019 campaign covered seven communes but was estimated to have achieved lower coverage levels (Figure 3, ranging from 5–60%). In the 2018 campaign, we used post-vaccination coverage transects to estimate vaccination coverage, but we were unable to do this in 2019 given the continuous delivery strategy. In addition, in Andasibe in 2018, coverage estimates were based on a single transect resulting in more uncertainty. However, coverage estimates from the transects in 2018 were consistent with the HDR-based estimates (for both Andasibe and Moramanga, transect-based estimates fell within the range of the HDR estimates). We also back-calculated HDRs given our vaccination coverage estimates, and these were similar to the HDRs calculated

from the household survey (15.7–32.8 for Andasibe compared to 21.7 in a rural community and 17.8–21.2 for Moramanga Ville compared to 17.2 in an urban community).

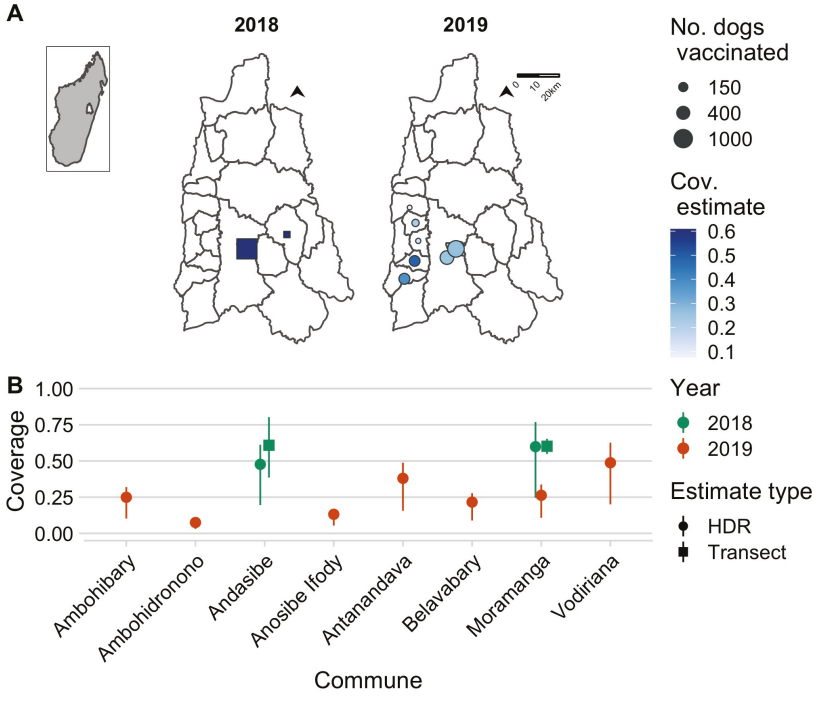

Figure 3. Estimates of coverage achieved by the 2018 and 2019 campaigns. (**A**) The commune-level numbers of dogs vaccinated (size of points) and the associated coverage estimates (color of points) for the year 2018 (squares, estimated using post-vaccination transects) and 2019 (circles, estimated using a human-to-dog ratio (HDR) of 19.5, based on a recent household survey in the Moramanga District). The inset shows the location of the Moramanga District in Madagascar. (**B**) A comparison of coverage estimates by location and by method of estimation (shape of points correspond to post-vaccination transects vs. HDR-based estimates); for transect-based estimates, the line range shows the 95% exact binomial confidence interval, while for the HDR-based estimates, the line range shows the range of coverage estimates assuming an HDR range of 8–25 according to estimates from the literature.

3.4. Dog Demography and Simulating Vaccination Strategies

Demographic data from vaccinated dogs showed a population pyramid with a large base, indicative of a fast-growing population, and with a male bias (approximately 60% male, Figure 4A). We fit these data to an age-structured model and were able to generate parameter estimates, which resulted in stable age distributions consistent with the data (Figure 4B). We filtered parameter estimates that are consistent with a growing population, resulting in mean adult annual survival probability of 0.77 (95% quantile: 0.59–0.97). We used estimates of fertility (on average, 1.09, 95% quantile: 0.82–1.41) to back-calculate pup survival, which ranged from 0.34 to 0.68. We found that, given these demographic parameters, annual campaigns that target dogs of all ages result in rapid decline in vaccination coverage between campaigns, largely due to rapid turnover of the dog population (compared to the impact of waning immunity assuming a discrete 3-year period of vaccine immunity, evident in the additional dip at year 3, Figure 4D). Continuously targeting 70% of the puppy population for vaccination while unable to achieve the peak coverage consistently reached coverage of about 50% of the dog population. A combined strategy

maintains the highest and most temporally stable levels of coverage close to the target of 70%.

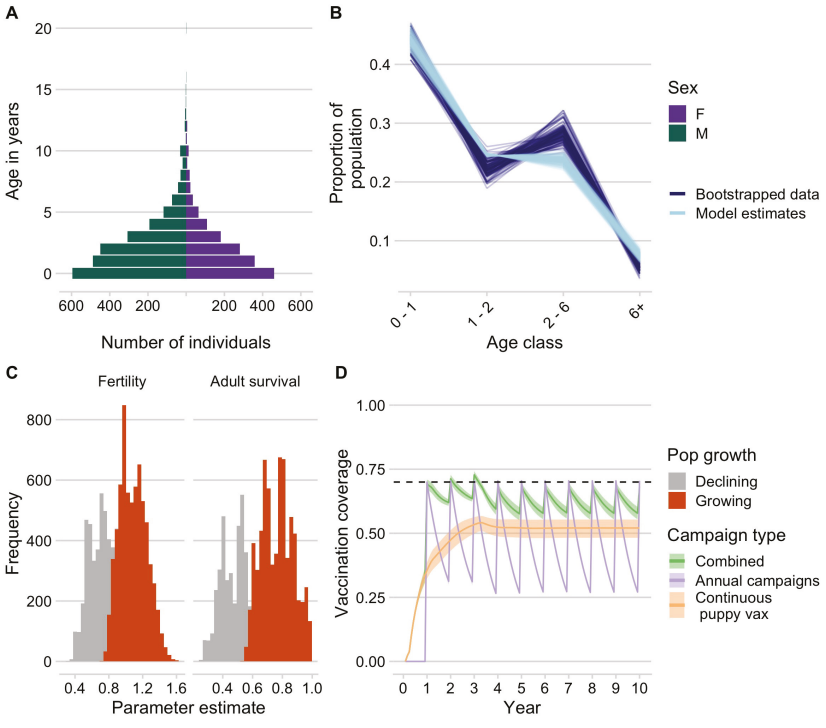

Figure 4. Dog demography and implications for campaign strategies. (**A**) The age pyramid for vaccinated dogs by sex. (**B**) Bootstrapped estimates of the proportion of the population in each age class from the age data (dark blue) compared to estimates from the demographic models fit to these data (light blue). (**C**) Parameter estimates for annual fertility rates and adult survival probability, with estimates highlighted in orange showing the parameter estimates that result in positive population growth. (**D**) Simulated vaccination coverage (N = 1000) using the demographic parameters from (**C**) in a hypothetical commune with 1000 dogs for three different campaign strategies: (1) annual vaccination campaigns targeting dogs of all ages (purple), (2) routine vaccination of puppies at 3 months of age, and (3) a combined strategy with campaigns annually and continuous puppy vaccination in between campaigns.

4. Discussion

Through vaccination campaigns implemented in Moramanga District of Madagascar, we saw a high demand for vaccination from dog owners and found that dogs were accessible and able to be handled safely and efficiently for parenteral vaccination at a reasonable cost (between 1.3–1.8 USD per animal vaccinated). We found that providing the vaccine at no direct cost to dog owners will be critical to achieving sufficient coverage, as even with nominal fees, a significant proportion of owners indicated that they would no longer vaccinate their animals. A static point vaccination strategy achieved higher coverage over a shorter time period in 2018 compared to dog vaccinations conducted by the district veterinarian as part of routine veterinary service provision in 2019. However, it came at a higher cost per animal vaccinated, was more limited in geographic scope, and required more resources in terms of personnel. In addition, in the rural setting of Andasibe, the static point campaign strategy achieved lower coverage, reflecting less accessible,

hard-to-reach human communities in this location. Based on the lessons learned through these campaigns, in particular, the observation that puppies were relatively easy to handle for vaccinators and owners, we found that continuous vaccination, targeting puppies in particular, may be an effective way to maintain vaccination coverage levels given the high turnover in dog populations.

There were several limitations to our analyses. Owner reports of willingness to pay, age of animals, and distance travelled to the vaccination point likely all suffer from recall bias and uncertainty. Owner-based estimates of age are very coarse, but given the broad age classes we used, they likely are of sufficient precision to capture broad patterns in age structure. However, the ages of the animals brought to vaccination points may not be representative of the age structure of the underlying population. Previous work has shown that, in general, puppies (individuals <1 year) tend to have lower vaccination coverage than adults [30–33]. Additionally, we did not vaccinate animals less than 1 month of age, which may explain why we sometimes estimated declining populations (as part of the age class of 0–1 years was not captured by the data). Despite these issues, our analyses based on these data are consistent with previous findings from sub-Saharan Africa that demonstrate male-biased populations, skewed towards puppies, and with high mortality in the first year of life [22,34].

Willingness-to-pay studies have been done previously for rabies vaccination and have consistently shown that cost recovery is minimal given the price dog owners are willing to pay for vaccination [35–38]. In fact, owners generally overstate the amounts they are willing to pay when compared to observed practice [39], and thus, our analysis could underestimate the impact of charging owners on coverage reductions. Similarly, distance to campaign points has been identified as a barrier to vaccination, and in most cases, owners report traveling less than 1 km to reach a vaccination point [32,40]. In the context of Madagascar, these findings are of particular relevance, as animal vaccinations can be mandated by the government but at a cost to animal owners (for example, for the anthrax vaccine in cattle). Importantly, dog owners in the district do believe that vaccination can prevent rabies transmission [21] and, given the observed demand during the campaigns, are amenable to vaccination of their animals. Our results confirm that implementing dog rabies vaccinations as a public health measure and removing as many barriers as possible to dog vaccination will be important to the success of control programs.

We used HDRs, which can be sensitive to underlying estimates of the human population and the spatial scale of estimation [15,41]. However, we used HDR estimates from a recent household survey study in the district and found these to give coverage estimates consistent with those from post-vaccination transects. Finally, in our vaccination model, we made several simplifying assumptions: we assumed that the protective effect of vaccination is lost after three years (simulating vaccination with a long-lasting vaccine such as Nobivac), but we also assumed that vaccinated dogs that survive to the following year are revaccinated in subsequent campaigns (effectively assuming boosting per manufacturer recommendations for Rabisin). Overall loss of vaccine-induced immunity plays a lesser role in declining vaccination coverage given the high population turnover in this context. In our age-structured models, we also do not account for population-carrying capacity, likely resulting in overestimates of growth of the dog population.

One key aspect that we do not consider is potential feedback loops between vaccination and demography. Estimates of the effects of vaccination on dog demography are mixed [22], but vaccination may increase dog survival [42]. If dog population growth is driven by survival, then this could mean that increased vaccination results in increased population growth. However, if growth is driven more by demand from human communities, then vaccination could stabilize the population and reduce population turnover. Improving dog population management, encouraging responsible pet ownership practices, and increasing veterinary services could all complement vaccination efforts but have not been demonstrated to result in meaningful rabies control without parallel dog vaccination [34].

Our estimates of costs per animal vaccinated are in line with recent estimates from other countries [43], although these are likely underestimates given the donation of time and resources by the organizations and individuals involved (i.e., costs associated with international volunteers including airfare and visa costs as well as costs of transportation donated to the campaign). We also did not include costs of pre-exposure prophylaxis as all of our volunteers and vaccinators had been vaccinated prior to the campaigns. Both pre- and post-exposure vaccines should however always be readily available for vaccinators and should be included in vaccination program budgets. While the volunteer-led effort resulted in significant financial and personnel resources being devoted to the campaign, costs were higher overall and per animal vaccinated. In addition, NGO- and volunteer-based campaigns may be difficult to sustain given unpredictable funding, time commitments, and turnover in staff [44]. For volunteer-based efforts, focusing on local volunteers (i.e., veterinary students) may be a more cost-effective strategy. However, similar to international volunteers, volunteers require subsistence during vaccination campaigns when not based in the communities where they study or live. Although we included costs of implementing transect-based coverage estimates, these were negligible (less than 0.10 USD per dog vaccinated), in line with recent studies that have shown that this strategy is a cheap and effective way to estimate coverage [15,16].

Dog vaccination delivered by the district veterinarian was less costly, with the majority of the costs directly related to vaccination. In settings with high dog ownership, moving towards community-based vaccination strategies could be an effective way to achieve sufficient and consistent coverage, particularly in hard-to-reach communities. During our campaign, we found that puppies (aged approximately 1–6 months) were easier to handle compared to adult dogs for both vaccinators and owners (see puppies picutred in basket in Figure 1). Puppy vaccination could be carried out by local officials embedded in communities (along the lines of community health workers who may not have full veterinary qualifications), especially given recent findings on the thermotolerance of rabies vaccines and locally manufactured methods for maintaining temperatures required for sustained vaccine storage (up to 3 months) [45]. Incentivizing vaccinators appropriately will be a key challenge, as currently providing no-cost rabies vaccination is not seen as part of routine duties for district veterinarians or for livestock officers. Implementing dog vaccination alongside government-mandated livestock vaccination campaigns may also be a strategy to scale up vaccination efforts at relatively low cost. Expanding veterinary services across the country and relieving financial pressures on veterinarians and animal health workers through appropriate compensation could greatly improve veterinary services across Madagascar [12].

Overall, our results suggest that dog vaccination is a feasible strategy for controlling canine rabies in Madagascar. However, rabies vaccination must be recognized as a public good. Removing barriers for dog owners and incentivizing veterinarians and other animal health workers to implement vaccination will be key to long-term campaign success. Borrowing strategies from human vaccination efforts, i.e., deploying community health workers, could be a way to deliver vaccinations and to reduce costs in the hardest-to-reach communities. In addition, refining vaccination strategies to local contexts and using improved tools and systems, such as mobile phone-based data collection, could improve efficacy and coverage levels reached [17,46]. To monitor the success of these campaigns, it will be critical to develop efficient and effective methods to estimate vaccination coverage and to measure their impact on reducing rabies incidence through robust surveillance [44,47]. With limited chances for reintroduction from outside the island, implementing community-based mass dog vacciantion campaigns could be a path forward for Madagascar to reach ZeroBy30.

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Informed Consent Statement: Prior to vaccination, verbal informed consent was obtained from animal owners, and owners could opt out of answering any questions or services provided. No personally identifiable information was collected at any point during the campaigns.

Data Availability Statement: All data and code are archived at https://doi.org/10.5281/zenodo.4663084, accessed on 30 March 2021 and available at https://github.com/mrajeev08/mora_vax, accessed on 30 March 2021.

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