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RESEARCH ARTICLE

Molecular Detection and Epidemiology of *Dirofilaria* spp. and *Acanthocheilonema reconditum* in Companion Animals from Central Punjab, Pakistan

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Abstract

Filarial nematodes including *Dirofilaria immitis*, *Dirofilaria repens*, and *Acanthocheilonema reconditum* are vector-borne parasites of veterinary and zoonotic significance, particularly in tropical and subtropical regions. Dogs and cats serve as reservoirs, sustaining parasite transmission in endemic zones. Despite their relevance, these filarial parasites have not been genetically characterized in Pakistan. This study aimed to determine molecular prevalence, assess associated risk factors and perform genetic characterization of *D. immitis*, *D. repens*, and *A. reconditum* in dogs and cats from Faisalabad and Lahore. A total of 400 blood samples were collected from both host species. PCR targeting the SS rRNA gene was performed, followed by sequencing of selected amplicons. Epidemiological data were gathered through structured questionnaires. Sequence analysis was conducted using ClustalX, BioEdit and MEGA X. The overall prevalence was 23.25%, with *A. reconditum* (12.75%) most prevalent and confined to dogs. *D. immitis* (6.5%) and *D. repens* (4.0%) were found in both species, more commonly in dogs. Infections were significantly associated with stray status, outdoor exposure, poor health and vector density. Sequences showed 98-99.5% similarity to global isolates. Phylogenetic analysis confirmed clustering within respective species clades. This study provides the first molecular insight into canine and feline filarial infections in Pakistan and highlights the need for expanded surveillance and vector control strategies.

Keywords: *Dirofilaria* spp., *Acanthocheilonema reconditum*, Epidemiology, Prevalence, Dogs, Cats, Pakistan

INTRODUCTION

Filarial nematodes transmitted by blood-feeding vectors are globally distributed parasites affecting a wide range of mammalian hosts, including domestic animals and humans. In companion animals, filarial infections are often chronic, underdiagnosed and associated with substantial veterinary and public health implications. The transmission and persistence of these parasites are influenced by various factors such as climate, vector density, animal movement and access to veterinary care. In regions with favorable ecological conditions, especially where mosquito vectors thrive, filarial diseases have emerged or re-emerged with increasing frequency [1,2]. Despite their significance, in many endemic regions like Pakistan, data on the true burden of filarial infections in pets remain scarce, particularly using molecular tools that offer superior diagnostic accuracy compared to conventional methods. Among the most clinically important filarial nematodes are *Dirofilaria immitis* and *Dirofilaria repens*, which are responsible for heartworm disease and subcutaneous dirofilariosis, respectively [3]. *D. immitis* resides in the pulmonary arteries and right heart chambers, often leading to severe cardiopulmonary dysfunction in dogs and occasionally in cats. *D. repens*, though less pathogenic, is associated with subcutaneous nodules, dermatitis and conjunctival infections and holds growing zoonotic importance. The distribution of these parasites varies across regions. *D. immitis* is endemic in the Americas, Southern Europe, Southeast Asia and parts of the Middle East, including reports from Iran, Türkiye and India [4,5]. Similarly, *D. repens* has expanded rapidly in Europe with endemicity confirmed in Italy, France, the Balkans and parts of Eastern Europe [6]. In Asia, both parasites have been reported in stray and owned dogs in China, India, Sri Lanka and Thailand, with prevalence

ranging from 2% to over 40% depending on the diagnostic method and host environment [7,8]. However, in Pakistan, published studies remain limited in scope and are largely reliant on microscopy or serological methods, which may underreport true infection rates.

Acanthocheilonema reconditum is another filarial parasite frequently found in dogs, though it is generally considered to be of low pathogenicity. It is transmitted by fleas and lice rather than mosquitoes and is often encountered as a confounding factor in heartworm diagnosis due to overlapping microfilarial morphology. While *A. reconditum* has been detected in countries like Italy, United States, Brazil and Sri Lanka, reports from South Asia remain sporadic and incomplete [9,10]. Although usually asymptomatic, there are increasing reports of co-infection with *Dirofilaria* spp., raising concerns about its epidemiological role and the possibility of misdiagnosis in mixed infections. Molecular tools have become essential to distinguish *A. reconditum* from other filarial species, particularly in endemic regions where mixed infections are likely. In Pakistan, this parasite remains largely neglected in surveillance studies, despite a high population of unmonitored dogs and limited routine veterinary care.

Molecular diagnostics, particularly PCR-based assays, have revolutionized the detection and identification of filarial nematodes by enabling species-level discrimination based on conserved genetic markers such as 18S rRNA and mitochondrial CO1 [9,11]. These tools have revealed previously undetected cases and co-infections in both pets and wildlife, offering greater sensitivity and specificity than microscopy. Risk factor based molecular epidemiological studies are especially valuable as they allow for stratification of prevalence by host, geography, environmental exposure and management practices. The present study aimed to determine the molecular prevalence and associated risk factors of *D. immitis*, *D. repens* and *A. reconditum* in domestic dogs and cats from two major districts of central Punjab, Pakistan, namely Faisalabad and Lahore, using PCR and statistical modeling. This is the first comprehensive investigation combining molecular diagnostics with risk factor analysis for canine and feline filariasis in Pakistan and its findings will contribute significantly to local disease mapping and the development of targeted vector control, preventive management and surveillance strategies.

Material and Methods

Informed Consent and Ethical Approval

Prior to sample collection, informed consent and permission were taken from the respective pet owners, who were briefed about the study objectives. Sampling was carried out under sterile conditions by trained personnel and paramedical staff following ethical and biosafety protocols approved by the Institutional Biosafety and Bioethics Committee (IBC), University of Agriculture, Faisalabad, Pakistan.

Sample Collection

Sampling was conducted in two districts of central Punjab, namely Faisalabad and Lahore, over a two-year period from 2020 to 2022. A total of 400 blood samples were randomly collected from domestic dogs and cats, with an equal distribution of 200 samples per district to ensure adequate spatial representation for epidemiological mapping, rather than species-proportional sampling. A convenience sampling method was employed to obtain 3-5 mL of blood from each animal, targeting both canine and feline populations for the detection of microfilariae. Blood was drawn aseptically from the cephalic vein of stray animals as well as those presented at veterinary hospitals, commercial farms and livestock markets. Each sample was carefully labeled with relevant metadata including host species, date of collection and locality. All blood samples were preserved in EDTA-coated tubes and maintained at 4°C until further processing.

Questionnaire Administration

A structured questionnaire containing open and close-ended questions was used to collect relevant epidemiological information from animal owners and caretakers through interviews. These factors included gender, age, level of outdoor exposure, locality, density of vector breeding sites, availability of veterinary care, living status and health condition of the animals. Locality (urban, peri-urban, rural) was assigned based on the precise sampling site (owner's address or location where the stray animal was caught). The density of mosquito breeding sites was determined individually for each sample through on-site visual assessment and categorized as low, moderate, or high based on the presence of stagnant water, drainage conditions, surrounding vegetation, animal shelters, and owner-reported mosquito nuisance. Questionnaire items were developed after reviewing previous literature and incorporating field insights to ensure contextual relevance.

DNA Extraction and PCR-Amplification

Genomic DNA was extracted from all collected blood samples using the WizPrep gDNA Mini Kit (Wizbiosolutions, Korea), following the manufacturer's standardized protocol to ensure high-quality yield suitable for downstream applications. The extracted DNA was then subjected to polymerase chain reaction (PCR) to amplify the small subunit ribosomal RNA (SS rRNA) gene specific to *Dirofilaria* spp. and *A. reconditum* using newly designed genus and species-specific primer sets. For *Dirofilaria* spp., the primer pair DF: 5'TCGTCATTGCTGCGGTTA-3'

and DR: 3'-TTCGTTTCCGGGAAGCTG-5' was used to amplify a 493bp fragment, while for *A. reconditum*, the primer set ARF: 5'CAGGTGATGGTTTGATGTGC-3' and ARR: 3'-CACTCGCACTGCTTCACTTC-5' targeted a 348bp region. The annealing temperature for both primer sets were optimized at 53°C to ensure specific and efficient amplification. Primer specificity was verified in silico using NCBI Primer-BLAST to ensure correct target binding and to avoid non-specific amplification, and representative PCR products were confirmed by Sanger sequencing.

PCR amplification was carried out in a 20 µL reaction volume consisting of 10 µL of 2X-PCR master mix (Thermo Scientific), 1µL of each forward and reverse primer, 5 µL of template DNA and 3µL of nuclease-free water. The thermal cycling conditions included an initial denaturation at 94°C for 5 min, followed by 40 amplification cycles of denaturation at 94°C for 40 seconds, annealing at 53°C for 30 sec and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR amplicons were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized using a Bio-Rad gel documentation system.

The presence of distinct DNA bands of expected sizes (493bp for *Dirofilaria* spp. and 348bp for *A. reconditum)* confirmed successful amplification. To prepare samples for sequencing, PCR-positive amplicons for each parasite were excised and purified from the gel using the FavorPrep GEL/PCR Purification Kit (Favorgen Biotech, Taiwan) according to the manufacturer's instructions. The purified DNA products were then sent to Lab Genetix, Lahore for paired-end Sanger sequencing to confirm species identity and enable downstream molecular analysis.

Sequence and Phylogenetic Analysis

The obtained nucleotide sequences of *Dirofilaria* spp. and *A. reconditum* were assembled and edited using PREGAP and GAAP4 of staden package (Version 2.0). A nucleotide query for the similarity of related organisms was conducted on NCBI using BLASTn to confirm species identity and determine the closest matching sequences. High-similarity sequences from diverse hosts and geographical locations were subsequently retrieved in FASTA format for downstream analysis. Multiple sequence alignment of the obtained and downloaded sequences was performed using ClustalX software (Version 2.1) [12], allowing identification of conserved regions, point mutations and sequence divergence. Percent nucleotide identity and pairwise evolutionary distances between sequences were calculated using BioEdit software [13], which facilitated assessment of inter and intra-species genetic similarity. Phylogenetic analysis was conducted in MEGA X software using the neighbor-joining method with 1.000 bootstrap replicates and bootstrap values ≥70% were considered to indicate strong node support to infer evolutionary relationships between our sequences and previously published reference sequences.

Statistical Analysis

The association between filarial infection and potential risk factors was analyzed using multiple logistic regression, including age, gender, housing type, outdoor exposure etc. as predictor variables. Moreover, pair-wise odds ratio comparisons were performed between categories within each risk factor at 95% confidence interval using SAS (1998). In addition, to visually represent the spatial and host-related differences in parasite distribution, a heat map was constructed using R package (Version 4.5.1)

Results

Overall Prevalence of *Dirofilaria* spp. and *A. reconditum*

The overall molecular prevalence of filarial parasites in the sampled canine and feline population from central Punjab was 23.25%. Among the three species identified, *A. reconditum* was the most prevalent, followed by *D. immitis* and *D. repens*. Notably, *A. reconditum* was exclusively detected in dogs, with markedly higher prevalence in Faisalabad (30%) than in Lahore (21%). In contrast, *D. immitis* was found in both dogs and cats, with higher detection rates in dogs from Faisalabad (13%) and Lahore (9%) and lower but consistent prevalence in cats (2%) from both districts. *D. repens* showed a more uniform distribution, being detected in both hosts and regions i.e., 5% in dogs and cats from Faisalabad and slightly lower in Lahore with 4% in dogs and 2% in cats. District-wise comparison revealed that filarial infection was more prevalent in Faisalabad, particularly among dogs, which exhibited the highest combined infection rate (18%), followed by dogs from Lahore (13%). Cats showed relatively lower infection rates with 7% positivity in Faisalabad and 4% in Lahore. Overall, the dogs were more affected than cats and Faisalabad exhibited a higher endemicity of filarial parasites than Lahore. These spatial and host-related differences in parasite distribution are visually represented in *Fig. 1.* The heatmap demonstrates clear species-wise clustering by host and district, highlighting the dominant presence of *A. reconditum* in dogs and the comparatively moderate but widespread occurrence of *Dirofilaria* spp. across both hosts and locations. The bar graph alongside further underscores the higher overall burden of *A. reconditum*, indicating its potential epidemiological significance in the studied region.

Risk Factor Analysis

In Faisalabad, the prevalence of *Dirofilaria* spp. was 15.8% in females compared to 9.5% in males, though the difference was not statistically significant (OR=1.781; 95% CI=0.758-4.182). In Lahore, females also had a non-

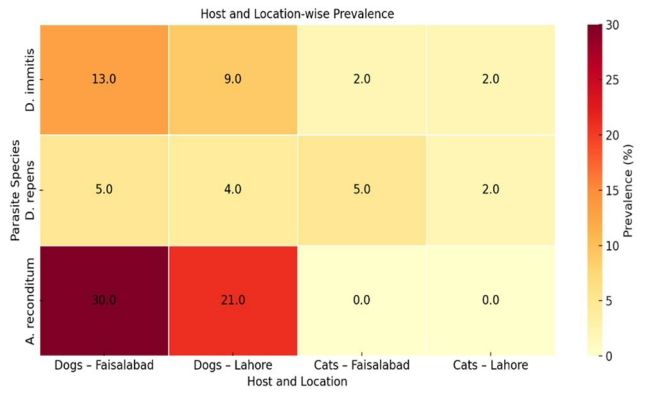

Fig 1. Combined heatmap and bar chart illustrating the prevalence of *D. immitis, D. repens*, and *A. reconditum* in dogs and cats from districts Faisalabad and Lahore, Pakistan. The heatmap shows the percentage of positive samples (n = 100 per group) for each parasite across different host-location combinations. The bar chart represents the overall number of positive cases (n = 400 total samples) for each parasite species

significantly (OR=1.424; 95% CI=0.504-4.0154) higher prevalence (9.6%) than males (7.0%). Age-wise analysis in Faisalabad revealed the highest prevalence in animals older than five years (18.2%; OR=2.444; 95% CI=0.779-7.670), followed by those aged 2–5 years (11.8%) and less than 2 years (8.3%). In Lahore, the same trend was observed with >5 years showing 12.7% prevalence (OR=2.406; 95% CI=0.666-8.685), while 2-5 years and <2 years had 8.0% and 5.7% prevalence, respectively. Outdoor exposure showed a significant association with infection. Animals with high exposure had a prevalence of 20.0% in Faisalabad (OR=3.500; 95% CI=1.072-11.419) and 15.5% in Lahore (OR=3.980; 95%; 95% CI=1.023-15.478). Locality-wise, animals from rural areas showed the highest prevalence in both districts: 20.6% in Faisalabad (OR=3.474; 95% CI=1.177-10.252) and 16.7% in Lahore (OR=3.900; 95% CI=1.107-13.739), while urban and peri-urban animals showed much lower rates. Regarding the density of vector breeding sites, animals from high-intensity areas had significantly higher prevalence in Faisalabad (20.0%; OR=3.813; 95% CI=1.184-12.270), while in Lahore the association was near significant (13.3%; OR=3.692; 95% CI=0.973-14.004). Animals without access to veterinary care were more infected than those with care, showing 18.1% prevalence in Faisalabad (OR=3.277; 95% CI=1.249-8.597) and 14.4% in Lahore (OR=4.474; 95% CI=1.404-14.249). Similarly, stray animals showed significantly higher infection than owned animals, with 18.3% prevalence in Faisalabad (OR=4.524; 95% CI=1.491-13.724) and 12.4% in Lahore (OR=3.215; 95% CI=1.010-10.229). Health status also influenced prevalence, with poor-condition animals showing the highest infection rates of 19.4% in Faisalabad (OR=3.082; 95% CI=0.472-5.228) and 14.0% in Lahore (OR=3.551; 95% CI=1.017-12.397), compared to 7.2% and 4.4% in healthy animals, respectively *(Table 1)*.

In Faisalabad, the prevalence of *A. reconditum* was 17.9% in females and 12.4% in males (OR=1.542; 95% CI=0.705-3.373). In Lahore, females also showed higher prevalence (12.3%) compared to males (8.1%), though the association remained nonsignificant (OR=1.580; 95% CI=0.608-4.102). Regarding age, animals older than five years had the highest prevalence in both districts i.e., 20% in Faisalabad (OR=2.25; 95% CI=0.770-6.569) and 16.4% in Lahore (OR=2.543; 95% CI=0.800-8.086), followed by animals aged 2-5 years (Faisalabad=15.3% and Lahore=9.3%) and <2 years (Faisalabad=10% and Lahore=7.1%). Outdoor exposure was significantly associated with infection in Faisalabad, where animals with high exposure had 24.6% prevalence (OR=3.591; 95% CI=1.225-10.529), while in Lahore the high-exposure group had a near-significant association (17.2%; OR=3.333; 95% CI=0.985-11.273). Locality also played a key role, as rural animals had the highest infection in both Faisalabad (22.1%; OR=3.113; 95% CI=1.130-8.577) and Lahore (20.8%; OR=4.052; 95% CI=1.293-12.693), in contrast to urban and peri-urban areas which showed much lower prevalence.

Animals from areas with high vector breeding intensity had significantly higher prevalence in Faisalabad (22.9%; OR=4.518; 95% CI=1.423-14.345) and Lahore (16.0%; OR=3.381; 95% CI=1.037-11.017), while low-intensity areas had only 6.2% and 5.3% prevalence respectively. Lack of veterinary care was significantly associated with infection, with animals without care showing 20.0% prevalence in Faisalabad (OR=2.388; 95% CI=1.034-5.5157) and 16.7% in Lahore (OR=3.466; 95% CI=1.285-9.350). Stray animals again showed higher infection rates than owned ones i.e., 20% in Faisalabad (OR=2.785; 95% CI=1.134-6.839) and 16.2% in Lahore (OR=4.394; 95% CI=1.422-13.577). Regarding health status, animals in poor condition had significantly higher prevalence of *A. reconditum* in both Faisalabad (25.4%; OR=4.352; 95% CI=1.502-12.606) and Lahore (17.5%; OR=3.659; 95% CI=1.181-11.338), while healthy animals had only 7.2% and 5.5% prevalence, respectively *(Table 2)*.

Sequencing and Phylogenetic Analysis

Pairwise alignment among obtained sequences revealed a high degree of identity within species, ranging from 99.0% to 99.5%, with 2-3 base pair differences. For *D. immitis*, two sequences namely DI-Pak1 (PV848760.1) from cat samples and DI-Pak2 (PV848759.1) from dog samples were analyzed. DI-Pak2 showed the highest similarity (99.58%) with isolates MN795071.1-MN795081.1 from dogs in France and also with Japanese isolates AB973230.1-AB973231.1. It additionally exhibited 99.55% similarity with Iranian isolates (MZ265271.1-MZ265283.1) and 99.12% with a Turkish isolate (PQ496477.1). DI-Pak1, derived from a feline host, showed 99.37% similarity with French and Japanese sequences, followed by 99.33%

Table 1. *Prevalence of Dirofilaria spp. infection in dogs and cats according to different epidemiological risk factors in Faisalabad and Lahore, Pakistan. Values represent number of positive animals out of those tested, prevalence (%), 95% confidence interval (CI), and odds ratio (OR) for the association between each risk factor category and infection status*

Variables	Category	District Faisalabad						District Lahore			
		Positive / Tested	Prevalence (%)	CI (95%)	Odds Ratio	P-Value	Positive / Tested	Prevalence %	CI (95%)	Odds Ratio	P-Value
Gender	Male	10/105	9.5	-	-	-	6/86	7.0	-	-	-
	Female	15/95	15.8	0.758-4.182	1.781	0.185	11/114	9.6	0.504-4.015	1.424	0.504
Age	<2 years	5/60	8.3	-	-	-	4/70	5.7	-	-	-
	2-5 years	10/85	11.8	0.556-1.260	1.474	0.506	6/75	8.0	0.387-5.314	1.435	0.589
	>5 years	10/55	18.2	0.779-7.670	2.444	0.126	7/55	12.7	0.666-8.685	2.406	0.180
Outdoor exposure	Low	4/60	6.7	-	-	-	3/68	4.4	-	-	-
	Medium	8/75	10.7	0.478-5.844	1.672	0.421	5/74	6.8	0.360-6.834	1.570	0.548
	High	13/65	20.0	1.072-11.419	3.500	0.038	9/58	15.5	1.023-15.478	3.980	0.046
Locality	Urban	5/72	6.9	-	-	-	4/82	4.9	-	-	-
	Peri-urban	6/60	10.0	0.431-5.143	1.489	0.529	5/70	7.1	0.386-5.817	1.500	0.558
	Rural	14/68	20.6	1.177-10.252	3.474	0.024	8/48	16.7	1.107-13.739	3.900	0.034
Density of vector breeding site	High	14/70	20.0	1.184-12.270	3.813	0.025	10/75	13.3	0.973-14.004	3.692	0.055
	Moderate	7/65	10.8	0.511-6.619	1.841	0.350	4/50	8.0	0.446-9.754	2.087	0.350
	Low	4/65	6.2	-	-	-	3/75	4.0	-	-	-
Availability of veterinary care	Yes	6/95	6.3	-	-	-	4/110	3.6	-	-	-
	No	19/105	18.1	1.249-8.597	3.277	0.016	13/90	14.4	1.404-14.249	4.474	0.011
Living status	Owned	4/85	4.7	-	-	-	4/95	4.2	-	-	-
	Stray	21/115	18.3	1.491-13.724	4.524	0.008	13/105	12.4	1.010-10.229	3.215	0.048
Health status	Good	5/69	7.2	-	-	-	4/91	4.4	-	-	-
	Fair	7/64	10.9	0.191-1.625	1.572	0.461	5/52	9.6	0.592-9.031	2.314	0.227
	Poor	13/67	19.4	0.472-5.228	3.082	0.044	8/57	14.0	1.017-12.397	3.551	0.047

identity with Iranian isolates (MZ265275.1MZ265283.1) and 98.53% with a mosquito-derived sequence (AF182647.1) from the USA.

For *D. repens*, DR-Pak1 (PV848917.1) from a cat and DR-Pak2 (PV848918.1) from a dog showed strong similarity to various global isolates. DR-Pak2 exhibited 99.58% similarity with sequences from a donkey (MN728180.1) and dog (MN728215.1) in Egypt, as well as with French isolates (MK495734.1, MK495735.1) and a Japanese human-derived isolate (AB973229.1). It also aligned 99.57% with French human-derived isolates (MZ427507.1MZ427510.1). DR-Pak1 showed 99.36% similarity with the same Egyptian isolates and 99.35% with the French human sequences but had lower identity (98.08%) with a Colombian dog isolate (OR029449.1).

For *A. reconditum*, two dog-derived isolates namely AR-Pak1 (PV878140.1) and AR-Pak2 (PV878141.1), were analyzed. AR-Pak1 showed highest similarity (99.03%) with an Indian isolate (GU593976.1) followed by 97.39% identity with a U.S. isolate (MZ468150.1) and 97.06% with Brazilian isolates (KX932116.1, KX932117.1). AR-Pak2 shared 98.70% similarity with the Indian reference sequence and showed lower identity with sequences from the USA (97.06%), Brazil (96.73%), Colombia (MZ473246.1) and Taiwan (AF217801.2).

Phylogenetic trees were constructed for *Dirofilaria* spp. and *A. reconditum* using the neighbor-joining method with 1000 bootstrap replicates. Distant nematodes including *Ascaris* sp. (JN256985.1) and *C. elegans* (EU196001.1) were used as an outgroup in trees to provide stable rooting and preserve correct tree topology. For *D. immitis*, both Pakistani sequences (PV848759.1 and PV848760.1) clustered together in a well-supported clade alongside isolates from Türkiye (PQ496477.1), Iran (MZ265274.1), Japan

(AB973230.1) and France (MZ275161.1), reflecting genetic conservation across Asian and Mediterranean

Table 2. *Prevalence of A. reconditum infection in dogs and cats according to different epidemiological risk factors in Faisalabad and Lahore, Pakistan. Values represent number of positive animals out of those tested, prevalence (%), 95% confidence interval (CI), and odds ratio (OR) for the association between each risk factor category and infection status*

Variables	Category	District Faisalabad					District Lahore				
		Positive / Tested	Prevalence (%)	CI (95%)	Odds Ratio	P-Value	Positive / Tested	Prevalence %	CI (95%)	Odds Ratio	P-Value
Gender	Male	13/105	12.4	-			7/86	8.1	-	-	-
	Female	17/95	17.9	0.705-3.373	1.542	0.277	14/114	12.3	0.608-4.102	1.58	0.347
Age	<2 years	6/60	10.0	-	-	-	5/70	7.1	-	-	-
	2-5 years	13/85	15.3	0.580-4.550	1.625	0.355	7/75	9.3	0.404-4.429	1.338	0.633
	>5 years	11/55	20.0	0.770-6.569	2.25	0.138	9/55	16.4	0.800-8.086	2.543	0.113
Outdoor exposure	Low	5/60	8.3	-	-	-	4/68	5.9	-	-	-
	Medium	9/75	12.0	0.474-4.738	1.5	0.489	7/74	9.5	0.466-5.984	1.671	0.429
	High	16/65	24.6	1.225-10.529	3.591	0.019	10/58	17.2	0.985-11.273	3.333	0.052
Locality	Urban	6/72	8.3	-	-	-	5/82	6.1	-	-	-
	Peri-urban	9/60	15.0	0.648-5.806	1.941	0.235	6/70	8.6	0.421-4.950	1.443	0.559
	Rural	15/68	22.1	1.130-8.577	3.113	0.028	10/48	20.8	1.293-12.693	4.052	0.016
Density of vector breeding site	High	16/70	22.9	1.423-14.345	4.518	0.010	12/75	16.0	1.037-11.017	3.381	0.043
	Moderate	10/65	15.4	0.822-9.349	2.772	0.100	5/50	10.0	0.502-7.736	1.972	0.330
	Low	4/65	6.2	-	-	-	4/75	5.3	-	-	-
Availability of veterinary care	Yes	9/95	9.5	-	-	-	6/110	5.5	-	-	-
	No	21/105	20.0	1.034-5.5157	2.388	0.041	15/90	16.7	1.285-9.350	3.466	0.014
Living status	Owned	7/85	8.2	-	-	-	4/95	4.2	-	-	-
	Stray	23/115	20.0	1.134-6.839	2.785	0.025	17/105	16.2	1.422-13.577	4.394	0.010
Health status	Good	5/69	7.2	-	-	-	5/91	5.5	-	-	-
	Fair	8/64	12.5	0.565-5.912	1.828	0.313	6/52	11.5	0.649-7.750	2.243	0.201
	Poor	17/67	25.4	1.502-12.606	4.352	0.006	10/57	17.5	1.181-11.338	3.659	0.024

Fig 2. Neighbor joining tree showing evolutionary relationship among obtained sequences of SS rRNA gene of *Dirofilaria* spp. with closely related sequences

populations. For *D. repens*, sequences PV848917.1 (cat) and PV848918.1 (dog) formed a tight cluster that aligned closely with the Japanese human isolate AB973229.1 and the Egyptian dog isolate MN728215.1 suggesting intra-species uniformity and potential zoonotic relevance *(Fig. 2)*. In the case of *A. reconditum*, sequences PV878140.1 and PV878141.1 clustered within a distinct, well-supported clade indicating close genetic relatedness and regional specificity. This clade was phylogenetically close to the Indian isolate (GU593976.1) highlighting limited divergence within South Asian populations. Pakistani isolates were clearly distinct from those originating in Brazil, Colombia and Taiwan, which formed separate clades suggesting geographical structuring among global *A. reconditum* lineages *(Fig. 3)*.

Discussion

This study represents the first molecular epidemiological investigation to assess the prevalence and risk factors associated with *Dirofilaria* spp. and *A. reconditum* in domestic dogs and cats from selected districts of central

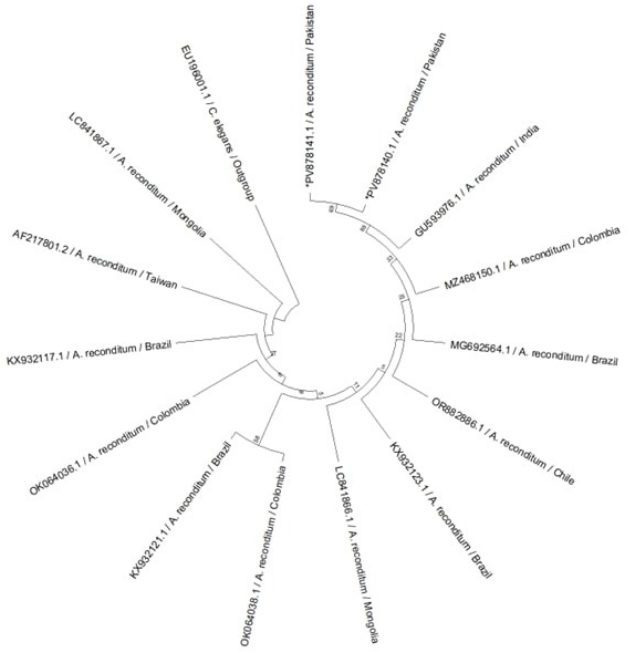

Fig 3. Neighbor joining tree showing evolutionary relationship among obtained sequences of SS rRNA gene of *A. reconditum* with closely related sequences

Punjab, Pakistan. A combined prevalence of 23.25% was recorded for all three filarial species, with *A. reconditum* (12.75%) being the most prevalent, followed by *D. immitis* (6.5%) and *D. repens* (4.0%). Notably, *A. reconditum* was detected exclusively in dogs, whereas *D. immitis* and *D. repens* were found in both dogs and cats, albeit with lower prevalence in feline hosts. This observation aligns with data from the USA and Poland, where *D. immitis* infection is significantly lower in cats, because felines are atypical hosts-larval development is frequently arrested, adult worms have a shorter lifespan, and microfilaremia is transient or absent, leading to reduced detectability and lower overall prevalence [14-16]. Similarly, the higher prevalence of *A. reconditum* and *D. repens* compared to *D. immitis* parallels findings from India, particularly in tropical and subtropical regions where these species dominate [17,18].

Host-related factors showed significant influence on filarial prevalence. Dogs exhibited markedly higher infection rates compared to cats, which reflects their role as primary hosts for *D. immitis* and their increased exposure to vectors due to outdoor activity. Risk was significantly elevated among stray dogs, animals in poor health condition and those lacking veterinary care. These patterns mirror findings from Iran, where *D. immitis* prevalence reached as high as 51.4% among stray dogs in Gilan province, particularly in environments characterized by outdoor exposure, high humidity and insufficient preventive measures [19]. In our study, animals sampled from rural and peri-urban areas with dense mosquito breeding sites showed higher odds of infection, which further supports the role of ecological drivers in transmission.

Environmental and geographical variables emerged as critical determinants of filarial burden. Animals in rural and peri-urban settings were at significantly higher risk compared to urban areas, underscoring the influence of sanitation, vector abundance and access to veterinary care. These findings resonate with global surveillance data from the USA, where the prevalence of *D. immitis* is notably higher in southern regions with warm, humid climates like Texas and Florida, compared to cooler states [14]. In Iran, regional differences in temperature, precipitation and humidity were strongly associated with infection prevalence, highlighted by high rates in Ahvaz and Meshkinshahr and lower prevalence in drier, cooler regions such as Hamadan and Tabriz [19].

Age-wise distribution revealed a trend of increasing infection with age, particularly among adult dogs (>5 years), indicating cumulative vector exposure over time. This is consistent with Iranian studies where dogs aged 3-15 years harbored the highest filarial burdens [20,21]. However, the potential for occult infections, especially with *D. immitis*, must be acknowledged. Up to 30% of dogs may carry adult worms without circulating microfilariae due to pre-patent stages, unisexual infections or immune-mediated clearance [14,17]. This limitation highlights the superior diagnostic accuracy of PCR-based molecular techniques, which were used in the current study and successfully detected infections regardless of blood microfilariae status.

Sequencing of selected PCR-positive amplicons further confirmed species identity and provided insights into genetic similarity and phylogeographic structure. Two *D. immitis* sequences (PV848760.1/cat and PV848759.1/dog) showed 99.58% similarity with French canine isolates (MN795071.1-MN795081.1) and closely clustered with sequences from Iran, Japan and Türkiye in the phylogenetic tree. Similarly, *D. repens* sequences (PV848917.1/cat and PV848918.1/dog) demonstrated 99.58% identity with isolates from Egypt and France and formed a robust clade with human and canine isolates from Japan and Europe, suggesting possible zoonotic potential. For *A. reconditum*, dog-derived sequences (PV878140.1 and PV878141.1) showed highest similarity with an Indian isolate (GU593976.1) and clustered closely in a well-supported clade distinct from those of Brazilian, Colombian and Taiwanese origin. These phylogenetic findings suggest limited intra-species variation within regional populations and underscore the genetic proximity of Pakistani strains to other South Asian isolates.

The phylogenetic analysis based on the SS rRNA gene provided strong support for species level identification,

although limited resolution for intraspecific diversity. High sequence similarity (>99%) within each species and well-supported bootstrap values in NJ trees validated the reliability of our molecular data. However, for finer-scale population genetics and haplotype resolution, future studies should target more variable loci such as ITS2 and CO1 genes. Studies from India have previously shown significant ITS2 variability among *A. reconditum* isolates from different regions [17] suggesting possible cryptic diversity that could also exist in Pakistani populations.

Our results also hold substantial public health relevance due to the zoonotic potential of *D. immitis* and *D. repens*. Human cases of subcutaneous and ocular dirofilariasis due to *D. repens* have been reported in Europe and Asia, often mimicking malignancies and leading to surgical misdiagnoses [6]. Likewise, *D. immitis* has been implicated in pulmonary coin lesions that are radiologically mistaken for tumors, sometimes resulting in unnecessary lung resections [5]. Given the presence of zoonotic species in both feline and canine hosts in our study, regular screening, preventive treatment and mosquito control should be emphasized, especially in endemic regions like central Punjab.

Despite the strengths of molecular confirmation and regionally representative sampling, our study has limitations. The use of convenience sampling may introduce selection bias, and therefore the prevalence estimates should be interpreted cautiously as they may not fully represent the entire companion animal population. The reliance on blood-derived DNA may not detect pre-patent or occult infections with very low parasitemia. Seasonal trends in vector abundance were not tracked and wider geographical surveillance is needed to assess nationwide distribution. Nevertheless, the findings presented here provide the first molecular confirmation of *D. immitis*, *D. repens* and *A. reconditum* in dogs and cats from central Punjab and offer a robust platform for ongoing surveillance, molecular epidemiology and zoonotic risk assessment.

This study provides the first molecular evidence of *D. immitis*, *D. repens* and *A. reconditum* infections in dogs and cats from Pakistan. The overall prevalence of 23.25% highlights the active circulation of filarial parasites in the region, with *A. reconditum* being the most frequently detected species in companion animals. Risk factor analysis revealed that outdoor exposure, rural locality, poor health status, lack of veterinary care and stray living conditions significantly contributed to higher infection rates. These findings emphasize the need for regular screening, improved vector control measures and increased awareness among pet owners and veterinarians to mitigate the spread of these parasites. Given the zoonotic potential of *D. immitis* and *D. repens*, this study also underlines the public health importance of filariasis surveillance and the integration of a One Health approach in endemic regions. Future studies should aim to include larger sample sizes, diverse ecological zones and multilocus molecular analyses to further explore parasite diversity and transmission dynamics in Pakistan.

Declarations

Availability of Data and Materials: All the generated data are included in the manuscript.

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Authors Contributions: MKK: Conceptualization and methodology; ZA: Data Collection, Processing, data analysis, and writing; RZA and MKS: Revised and proof-read. All authors read and approved the final manuscript.

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