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Gastrointestinal parasitism in cattle and water buffaloes in Pakistan: prevalence, risk factor analysis, and molecular characterization of Haemonchus contortus

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Abstract

Purpose

Gastrointestinal parasites are a major constraint to livestock productivity in Pakistan. However, comprehensive epidemiological data for cattle and water buffaloes in the Malakand Division remain limited. This study aimed to assess prevalence, risk factors, and molecular characteristics of key parasites to support targeted control strategies.

Methods

A cross-sectional study was conducted from January to December 2022 across nine districts of the Malakand Division (Khyber Pakhtunkhwa, Pakistan). A total of 972 fecal samples (496 cattle, 476 water buffaloes) were analyzed using flotation, sedimentation, and McMaster techniques. Associations with risk factors were evaluated using statistical analysis. Molecular characterization of *Haemonchus contortus* was performed by PCR amplification of the ITS-1 region, followed by sequencing and Maximum Likelihood phylogenetic analysis.

Results

Overall parasite prevalence was 31.79%, significantly higher in cattle (36.49%) than in water buffaloes (26.89%). *H. contortus* was the dominant parasite (23.30%), followed by *Cooperia* spp. and *Oesophagostomum* spp. Younger animals (< 2 years) showed significantly higher prevalence than older animals (> 7 years; $p < 0.001$). Gender effects differed between species, with higher prevalence in female cattle and male buffaloes. Seasonal variation was significant at the population level, with higher prevalence in spring and summer ($p = 0.036$), while geographic variation was not significant. Water source was significantly associated with infection in buffaloes ($p = 0.021$). *Haemonchus* showed the highest egg counts, indicating strong transmission potential. Molecular analysis confirmed *H. contortus* identity, with high similarity (up to 100%) to Pakistani isolates and moderate variation with global sequences (≈ 98.60%).

Conclusion

Gastrointestinal parasitism represents a moderate but significant burden in the Malakand Division, with *H. contortus* as the dominant species. Age, host species, gender, season, and water source are key determinants of infection. Targeted control strategies focusing on high-risk groups, seasonal timing, and improved management practices are essential. Molecular findings provide a baseline for monitoring parasite diversity and supporting future control programs.

1. Introduction

Livestock, particularly cattle and water buffaloes, play a vital economic role in Pakistan. These animals provide essential resources including milk, meat, and draft power that support the livelihoods of millions of rural households [1]. Pakistan hosts one of the world's largest livestock populations, comprising approximately 49 million head of cattle and 41.2 million water buffaloes as of 2020 [2]. The livestock industry is a cornerstone of Pakistan's economy, contributing approximately 60% of agricultural GDP and 11% of overall GDP [3]. Ensuring the health and productivity of these livestock populations is critical for sustainable economic development and national food security. However, gastrointestinal parasitic diseases, particularly gastrointestinal nematodes (GINs), represent a major health constraint in cattle and water buffaloes throughout Pakistan. *Haemonchus contortus* is among the most prevalent and economically important GIN species affecting ruminants in this region [4, 5]. Infections with gastrointestinal parasites have profound negative impacts on animal health and productivity. Clinical manifestations include reduced feed intake, weight loss, decreased milk production, poor body condition, and mortality in severe cases [6, 7]. The economic burden of gastrointestinal parasitism is substantial, encompassing direct losses from reduced livestock productivity as well as indirect costs associated with treatment and control measures [8].For example, a recent survey documented *Haemonchus* species prevalence of 28.70% in livestock populations [9], highlighting the considerable disease burden in the region. Despite the economic significance of livestock production in Pakistan, comprehensive epidemiological data on gastrointestinal parasites remain limited, particularly for specific agricultural regions. Studies investigating the prevalence and risk factors for gastrointestinal parasitism in cattle and buffaloes within the Malakand division are particularly scarce [9, 10]. The Malakand division is a substantial agricultural region in northwestern Pakistan with significant cattle and water buffalo populations. Understanding the distribution and prevalence of gastrointestinal parasites within this specific region is essential, as parasitic infections exhibit considerable geographic variation influenced by climate, management practices, and local environmental conditions [11]. Multiple host and environmental factors have been identified as important determinants of gastrointestinal parasite prevalence in ruminant populations, including host species, age, sex, season, and geographical location [12] Identifying which risk factors are most significant in the Malakand division would enable development of targeted, cost-effective parasite control strategies tailored to local conditions. The primary objective of this study was to determine the prevalence of gastrointestinal parasites, with particular emphasis on molecular identification of *H.*

contortus, in cattle and water buffaloes within the Malakand division of Pakistan. Secondary objectives included evaluating potential risk factors influencing parasitic infection rates, namely host species, age, sex, season, and geographical location. The findings are intended to improve understanding of gastrointestinal parasitism in this important livestock production region and to provide evidence-based guidance for developing effective regional parasite control strategies.

2. Materials and methods

2.1. Study area

The study was conducted in the Malakand Division, located in the northern region of Khyber Pakhtunkhwa province, Pakistan. The division comprises nine administrative districts: Swat, Shangla, Buner, Malakand, Dir Upper, Dir Lower, Chitral Upper, Chitral Lower, and Bajaur, covering an approximate area of 32,007 km² with a human population of about 8.7 million. The region is characterized by heterogeneous topography, including mountainous terrain, river valleys, and agricultural plains, supporting extensive livestock farming systems based on grazing and mixed crop-livestock practices. Climatically, the area falls within a subtropical to temperate mountain climate zone, with marked seasonal variations that are relevant for parasite transmission. The mean annual temperature is estimated to range between 10°C and 30°C, with colder conditions in winter and warmer temperatures during summer. Annual precipitation is approximately 800–1200 mm, with peak rainfall occurring during spring (March-April) and monsoon-associated months, while the lowest precipitation is typically observed in late autumn. Relative humidity is highest during the summer months (June-September), creating favorable environmental conditions for the development and survival of infective stages of gastrointestinal parasites. In contrast, the colder and drier winter period (November-February) is generally less conducive to parasite transmission. These climatic and ecological conditions are considered key drivers of the seasonal dynamics of gastrointestinal parasitism in livestock populations in the study area (Fig. 1).

2.2. Study population and sampling design

A cross-sectional study was conducted to estimate the prevalence of gastrointestinal parasites in cattle and water buffaloes in the Malakand Division. A total of 972 fecal samples were collected, including 496 from cattle and 476 from water buffaloes, between January and December 2022. Sampling was performed across nine districts of the Malakand Division: Dir Lower, Dir Upper, Swat, Malakand, Buner, Chitral Lower, Chitral Upper, Bajaur, and Shangla. A stratified random sampling design was applied, with districts considered as strata. The number of samples collected from each district was allocated proportionally based on the estimated livestock population in each district. Within each district, farms and abattoirs were selected using simple random sampling. Individual animals were then randomly selected from eligible animals present at the time of sampling. The required sample size was determined using a standard formula for prevalence studies based on an expected prevalence of 30%, a confidence level of 95%, and a desired precision of 5%. Animals were eligible for inclusion if they were cattle or water buffaloes aged between 6 months and 10 years, clinically stable at the time of sampling, and had not received any anthelmintic treatment within the preceding 8 weeks. Animals were excluded if they fell outside the defined age range, had been recently treated with anthelmintics, were severely ill, could not be safely handled, lacked reliable treatment history, or if owner consent was not obtained.

2.3. Sample collection procedures

Fecal samples were collected directly from the rectum of live animals using disposable gloves to avoid cross-contamination. Approximately 5 g of fecal material was obtained from each animal and immediately placed into sterile plastic containers containing 10% buffered formalin for preservation of parasitic stages intended for morphological examination. Each sample was assigned a unique identification code, and associated metadata, including host species, age, sex, geographic origin (district and village), water source, and management practices, were recorded using a standardized data collection sheet and subsequently entered into a digital database (Microsoft Excel 2016). For molecular analyses, adult nematodes were collected from the abomasum of naturally infected animals during routine slaughter at selected abattoirs located in Dir Lower, Swat, and Dir Upper districts, representing major livestock-producing areas of the Malakand Division. The selection of abattoirs was based on accessibility and slaughter throughput. Recovered adult *Haemonchus* specimens were gently washed in sterile phosphate-buffered saline (PBS) to remove debris and host material, then individually transferred into sterile microcentrifuge tubes. Samples were stored at -20°C until DNA extraction. The storage duration prior to DNA extraction ranged from 2 to 4 weeks. Fecal samples and parasite specimens were transported to the laboratory under appropriate temperature-controlled conditions and processed within 5 days of collection.

2.4. Parasitological examination of fecal samples

2.4.1. Direct saline smear

Upon arrival at the laboratory, fecal samples were subjected to microscopic examination for the detection of parasitic elements. A direct saline smear was prepared by mixing a small quantity of feces (approximately 0.5 g) with a drop of physiological saline (0.85% NaCl) on a clean glass slide. The preparation was covered with a coverslip and examined under 10× and 40× magnifications using a compound microscope (Labo

America Inc., USA). This method was primarily used for the detection of protozoan trophozoites and cysts, as well as for preliminary screening of helminth eggs.

2.4.2. Fecal flotation technique

A flotation technique was performed using a saturated sodium chloride solution (specific gravity ≈ 1.20) to detect nematode and cestode eggs. Approximately 3 g of fecal material was homogenized in distilled water and filtered through a 250 µm mesh sieve into a centrifuge tube. The filtrate was centrifuged at 1000 × g for 5 minutes, and the supernatant was discarded. The sediment was resuspended in saturated NaCl solution and centrifuged again at 1000 × g for 5–6 minutes. A small volume from the surface meniscus was collected using a Pasteur pipette, transferred onto a glass slide, covered with a coverslip, and examined under 10× magnification.

2.4.3. Sedimentation and quantitative McMaster technique

Sedimentation was carried out following standard procedures [13] for the detection of heavier parasitic stages. Briefly, the fecal suspension was centrifuged, and the supernatant discarded. The sediment was stained with a drop of methylene blue and examined microscopically under 10× and 40× magnifications. Quantitative estimation of nematode egg burden was performed using the McMaster technique as described by Foreyt [14], with slight modifications. Briefly, 4 g of feces were homogenized in 56 mL of saturated sodium chloride solution to obtain a uniform suspension. The mixture was filtered through cheesecloth, and the filtrate was used to fill both chambers of a McMaster counting slide. After allowing the eggs to float for approximately 5 minutes, eggs within the counting grids of both chambers were counted under 10× magnification. Each sample was analyzed in duplicate, and the mean egg count was used for calculation.

2.4.4. Egg per gram (EPG) calculation and infection intensity classification

Egg counts were expressed as eggs per gram (EPG) of feces using the McMaster technique. The EPG values were calculated according to the standard formula: EPG = (total number of eggs counted in both chambers × 50). For each sample, counts from the two chambers were averaged to improve accuracy. In cases of high egg density, fecal suspensions were further diluted (1:2 or 1:4) and re-examined to avoid overestimation due to chamber saturation. Based on the obtained EPG values, the intensity of infection was categorized into four classes: light (50–799 EPG), moderate (800–1199 EPG), heavy (1200–1999 EPG), and very heavy (> 2000 EPG), in accordance with the classification scheme applied in the results section. This classification allowed for a standardized assessment of parasite burden and facilitated comparison of infection intensity across host species and risk factor categories.

2.5. Morphological identification of gastrointestinal parasites

Parasite identification was performed based on the morphological characteristics of eggs, oocysts, and cysts observed in fecal preparations. Identification was carried out using standard parasitological keys and comparative morphology, taking into account features such as size, shape, color, shell structure, and internal contents. Helminth eggs were identified primarily at the genus or group level, as differentiation at the species level is generally not reliable based on egg morphology alone. Strongyle-type eggs (including *Haemonchus*, *Cooperia*, and *Oesophagostomum*) were identified as oval, thin-shelled eggs containing multiple blastomeres. In contrast, *Trichuris* spp. eggs were readily distinguished by their characteristic barrel or lemon shape, with prominent bipolar plugs and a thick, smooth shell. Cestode eggs such as *Moniezia* spp. were identified based on their polygonal shape and the presence of a pyriform apparatus, while protozoan cysts (e.g., *Entamoeba* spp.) were recognized by their size, shape, and internal nuclear structures. Measurements were performed using an ocular micrometer when necessary to support identification. Morphological identification was used for preliminary classification of parasites, while species-level identification of *H. contortus* was confirmed through molecular analysis.

2.6. Molecular characterization of Haemonchus contortus

2.6.1. DNA extraction and PCR amplification

Genomic DNA was extracted from individual adult *Haemonchus* worms using a commercial DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The internal transcribed spacer 1 (ITS-1) region of ribosomal DNA was amplified by polymerase chain reaction (PCR). PCR reactions were carried out in a total volume of 25 µL containing 1 µL of template DNA (approximately 25–50 ng), 1 µL of each primer (final concentration 0.5 µM), 12.5 µL of 2× Taq PCR Master Mix (Qiagen, Germany), and nuclease-free water to volume. The PCR Master Mix contained Taq DNA polymerase, reaction buffer, $MgCl_2$, and deoxynucleotide triphosphates (dNTPs). Amplification was performed using the species-specific primers HcBotuF1 (5'-TGTCGAACACGAAACTCGTC-3') and HcBotuR2 (5'-TGTGTCTCTACCGCCCGAGT-3'), targeting the ITS-1 region. The expected amplicon size was approximately ~ 250 bp. Thermal cycling conditions consisted of an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were separated by electrophoresis on 1.5–2% agarose gels prepared in 1× TBE buffer and stained with ethidium bromide. A 100 bp DNA ladder was used as a molecular size marker, and bands were visualized under UV illumination. Among the samples identified as strongyle-positive by microscopic examination, 20 produced clear PCR amplicons of the expected size. Only samples yielding distinct and reproducible bands were retained for downstream molecular analyses.

2.6.2. PCR product purification and sequencing

PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) following the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop spectrophotometer by measuring absorbance at 260 and 280 nm. Only samples with DNA concentrations ≥ 20 ng/μL and acceptable purity ratios (A260/A280 ≈ 1.8-2.0) were selected for sequencing. Purified PCR products were subjected to bidirectional Sanger sequencing. Forward and reverse sequences were assembled and edited using BioEdit software (version 7.2.6.1) to obtain consensus sequences. Representative sequences generated in this study were submitted to the GenBank database under accession number PV599769.

2.6.3. Sequence analysis and comparison

Consensus sequences were compared with reference sequences available in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). Sequences showing high similarity (typically ≥ 98% identity) with published *H. contortus* ITS-1 sequences were considered as confirmed *H. contortus* isolates.

2.6.4. Phylogenetic analysis

Phylogenetic tree based on ITS-1 sequences was constructed using the Maximum Likelihood method (Tamura and Nei, 1993) with Tamura-Nei nucleotide substitution model, selected following jModelTest analysis as the best-fit model, implemented in DNAMAN software. Tree robustness was assessed by bootstrap analysis with 1000 replicates. These analyses allowed an evaluation of the genetic relationships and diversity among the *H. contortus* isolates in the context of previously reported sequences.

2.7. Ethical considerations

All procedures involving animals were conducted in accordance with established ethical standards for the care and use of animals in research. Fecal sampling by rectal collection, considered a minimally invasive procedure, was performed by trained personnel following standard veterinary practices to ensure animal welfare and minimize stress and discomfort.

Informed consent

was obtained from animal owners prior to sample collection. The study protocol was reviewed and approved by the Departmental Ethical Committee of the Department of Zoology, Abdul Wali Khan University Mardan, Pakistan (Reference No. ICE-202-05, approved on 11-12-2022; Approval No. 26 – 0023). All efforts were made to ensure that animals were handled humanely and that no unnecessary suffering occurred during the sampling procedures.

2.8. Statistical analysis

The prevalence of gastrointestinal parasites was calculated as the proportion of infected animals relative to the total number examined and expressed as a percentage with 95% confidence intervals (95% CI). Prevalence estimates were computed for the overall study population and stratified by host species, district, and sampling period (monthly and seasonal) to describe spatial and temporal variations. Associations between infection status (positive/negative) and potential risk factors were initially assessed using univariable analysis. Explanatory variables included host species (cattle versus water buffaloes), age category (< 2 years, 2–7 years, > 7 years), sex (male versus female), geographic location (district), season, and type of drinking water source (surface water, municipal water, well water, or stored rainwater). Categorical variables were compared using chi-square (χ^2) tests, and Fisher's exact test was applied when expected frequencies were less than 5. A p-value < 0.05 was considered statistically significant. To identify independent risk factors, multivariable logistic regression models were constructed. Variables with p-values < 0.05 in univariable analysis were included in the initial model. A backward stepwise selection procedure based on the Akaike Information Criterion (AIC) was used to obtain the final model. Model fit was assessed using the Hosmer–Lemeshow goodness-of-fit test, and potential model assumptions, including multicollinearity, were evaluated using variance inflation factors (VIF). Results of the final models are presented as odds ratios (OR) with 95% confidence intervals (95% CI). All statistical analyses were performed using R software (version 4.3.1; R Core Team, 2023), with relevant packages including *stats* and *epitools*.

3. Results

3.1. Relative prevalence of different gastrointestinal parasite genera and species

Among the 972 animals examined, 309 (31.79% ± 2.94%) were positive for at least one gastrointestinal parasite, including 181 cattle (36.49% ± 4.28% of 496 examined) and 128 water buffaloes (26.89% ± 3.92% of 476 examined). A total of ten gastrointestinal parasite genera were identified among the infected animals (Table 1). As presented in Table 1, *Haemonchus* spp. was the most frequently detected parasite across both host species, accounting for 22.10% of infected cattle, 25.00% of infected water buffaloes, and 23.30% of all infected animals. In cattle, *Oesophagostomum* spp. (12.71%) and *Entamoeba* spp. (11.60%) were also among the most commonly observed parasites. In water buffaloes,

Entamoeba spp. (10.16%), *Cooperia* spp. (10.94%), and *Ostertagia ostertagi* (9.38%) showed relatively high frequencies following *Haemonchus* spp. Overall, the most prevalent parasites among all infected animals were *Haemonchus* spp. (23.30%), followed by *Entamoeba* spp. and *Cooperia* spp. (11.00% each), and *Oesophagostomum* spp. (10.03%). Lower relative frequencies were observed for *Trichuris* spp. (7.44%), *Bunostomum phlebotomum* (8.09%), and *Nematodirus* spp. (8.41%). The distribution of parasite genera varied between cattle and water buffaloes, although strongyle-type nematodes were predominant in both host species. Statistical analysis indicated that the distribution of parasite genera differed significantly within infected water buffaloes (χ^2 test, $p < 0.001$), and in the combined infected population (χ^2 test, $p < 0.001$) (Table 1).

Table 1 Relative prevalence of different gastrointestinal parasite genera and species in cattle and water buffaloes

Parasites	Cattle positive to GI parasites (n=181)			Water buffaloes positive to GI parasites (n=128)			All animals positive to GI parasites (n=309)		
	Positive	Prevalence (% ± CI_{95})	P value	Positive	Prevalence (% ± CI_{95})	P value	Positive	Prevalence (% ± CI_{95})	P value
Entamoeba spp.	21	11.60 ± 2.35	0.05	13	10.16 ± 2.58	0.00*	34	11.00 ± 1.79	0.00*
Moniezia spp.	18	9.94 ± 2.23		11	8.59 ± 2.43		29	9.39 ± 1.67	
Haemonchus contortus	40	22.10 ± 3.03		32	25.00 ± 3.40		72	23.30 ± 2.44	
Trichuris spp.	14	7.73 ± 1.95		9	7.03 ± 2.25		23	7.44 ± 1.48	
Ostertagia ostertagi	19	10.49 ± 2.29		12	9.38 ± 2.55		31	10.03 ± 1.75	
Cooperia spp.	20	11.05 ± 2.33		14	10.94 ± 2.75		34	11.00 ± 1.79	
Bunostomum phlebotomum	18	9.94 ± 2.23		7	5.47 ± 2.00		25	8.09 ± 1.55	
Nematodirus spp.	16	8.84 ± 2.09		10	7.81 ± 2.39		26	8.41 ± 1.59	
Toxocara spp.	17	9.39 ± 2.17		12	9.38 ± 2.55		29	9.39 ± 1.67	
Oesophagostomum spp.	23	12.71 ± 2.47		8	6.25 ± 2.12		31	10.03 ± 1.75	

Abbreviations: CI_{95} = 95% confidence interval; * = statistically significant ($p < 0.05$).

3.2. Geographical variation in gastrointestinal parasite prevalence

Gastrointestinal parasite prevalence varied across the nine districts of the Malakand Division (**Table 2**). In cattle, the highest prevalence was observed in Chitral Upper, Bajaur, and Shangla (44.00% each), while the lowest prevalence was recorded in Dir Upper (26.67%). In water buffaloes, Buner showed the highest infection rate (42.31%), whereas Shangla exhibited the lowest prevalence (18.00%). When considering all animals combined, Buner again had the highest prevalence (41.67%), followed by Chitral Upper (35.00%) and Bajaur (34.00%), while Dir Upper showed the lowest overall prevalence (24.14%) (**Table 2**). Despite these variations, statistical analysis indicated that the differences in prevalence among districts were not significant for cattle ($\chi^2 = 3.84$, $df = 8$, $p = 0.21$), water buffaloes ($\chi^2 = 3.85$, $df = 8$, $p = 0.050$), or the combined population ($\chi^2 = 1.32$, $df = 8$, $p = 0.20$).

Table 2 Geographical variation in gastrointestinal parasite prevalence

Geographic	Cattle				Water buffaloes				All animals			
region	Total	Positive	Prevalence (% ± CI_{95})	P value	Total	Positive	Prevalence (% ± CI_{95})	P value	Total	Positive	Prevalence (% ± CI_{95})	P value
Dir (L)	60	18	30.00 ± 11.80	0.210	56	20	35.71 ± 12.73	0.050	116	38	32.76 ± 8.62	0.200
Dir (U)	60	16	26.67 ± 11.34		56	12	21.43 ± 10.87		116	28	24.14 ± 7.98	
Swat	60	17	28.33 ± 11.48		56	19	33.93 ± 12.57		116	36	31.03 ± 8.53	
Malakand	60	21	35.00 ± 12.31		56	10	17.86 ± 10.02		116	31	26.72 ± 8.17	
Buner	56	23	41.07 ± 13.18		52	22	42.31 ± 13.58		108	45	41.67 ± 9.41	
Chitral Lower	50	20	40.00 ± 13.86		50	11	22.00 ± 11.55		100	31	31.00 ± 9.12	
Chitral Upper	50	22	44.00 ± 14.00		50	13	26.00 ± 12.11		100	35	35.00 ± 9.50	
Bajaur	50	22	44.00 ± 14.00		50	12	24.00 ± 11.84		100	34	34.00 ± 9.40	
Shangla	50	22	44.00 ± 14.00		50	9	18.00 ± 10.45		100	31	31.00 ± 9.12	
Total	496	181	36.49 ± 4.28		476	128	26.89 ± 3.92		972	309	31.79 ± 2.94	

Abbreviations: CI_{95} = 95% confidence interval; P value reported for χ^2 test.

3.3. Temporal variation: Monthly prevalence patterns

Monthly variations in gastrointestinal parasite prevalence were observed throughout the study period (**Table 3**). In cattle, prevalence increased progressively from January (33.33%) to a peak in August (46.34%), followed by a decline to the lowest level in December (19.51%). In water buffaloes, prevalence showed less pronounced fluctuations, with a peak in March (35.00%) and a minimum in November (23.68%). When considering all animals combined, the highest prevalence was recorded in July and August (38.27%), while the lowest was observed in December (22.78%) (**Table 3**). Statistical analysis revealed a significant temporal trend in cattle ($\chi^2 = 3.32$, $p = 0.03$), indicating a seasonal increase in infection during warmer months. In contrast, no significant temporal trends were detected in water buffaloes ($\chi^2 = 1.33$, $p = 0.50$) or in the combined population ($\chi^2 = 1.32$, $p = 0.34$).

Table 3 Month wise prevalence patterns of cattle and water buffaloes in Malakand division

Months	Cattle				Water buffaloes				All animals			
	Total	Positive	Prevalence (% ± CI_{95})	P value	Total	Positive	Prevalence (% ± CI_{95})	P value	Total	Positive	Prevalence (% ± CI_{95})	P value
January	42	14	33.33 ± 14.27	0.03	40	7	17.50 ± 11.89	0.500	82	21	25.61 ± 9.57	0.03
February	42	16	38.10 ± 14.90		40	8	20.00 ± 12.43		82	24	29.27 ± 9.90	
March	42	17	40.48 ± 15.10		40	14	35.00 ± 14.54		82	31	37.80 ± 10.72	
April	41	18	43.90 ± 15.39		40	11	27.50 ± 13.86		81	29	35.80 ± 10.64	
May	41	16	39.02 ± 15.02		40	12	30.00 ± 14.04		81	28	34.57 ± 10.62	
June	41	17	41.46 ± 15.18		40	12	30.00 ± 14.04		81	29	35.80 ± 10.64	
July	41	18	43.90 ± 15.39		40	13	32.50 ± 14.29		81	31	38.27 ± 10.77	
August	41	19	46.34 ± 15.53		40	12	30.00 ± 14.04		81	31	38.27 ± 10.77	
September	42	17	40.48 ± 15.10		40	10	25.00 ± 13.67		82	27	32.93 ± 10.37	
October	41	11	26.83 ± 13.53		40	10	25.00 ± 13.67		81	21	25.93 ± 9.81	
November	41	10	24.39 ± 13.19		38	9	23.68 ± 13.51		79	19	24.05 ± 9.68	
December	41	8	19.51 ± 12.15		38	10	26.32 ± 13.76		79	18	22.78 ± 9.52	
Total	496	181	36.49 ± 4.28		476	128	26.89 ± 3.92		972	309	31.79 ± 2.94	

Abbreviations: CI_{95} = 95% confidence interval; P value reported for χ^2 test for trend

3.4. Seasonal prevalence patterns

Seasonal analysis revealed clear temporal patterns in gastrointestinal parasite prevalence (Table 4). In cattle, prevalence was highest during spring (42.17%) and summer (42.68%), followed by a decline in autumn (33.73%) and winter (28.92%). A similar pattern was observed in water buffaloes, with the highest prevalence recorded in spring (31.25%) and a gradual decrease toward winter (23.08%). When all animals were considered collectively, prevalence was higher in spring (36.81%) and summer (36.11%) compared to autumn (29.45%) and winter (26.09%) (Table 4). Statistical analysis demonstrated a significant seasonal effect in the combined population (χ^2 test, $p = 0.03$), whereas no significant seasonal differences were detected when cattle ($p = 0.17$) and water buffaloes ($p = 0.24$) were analyzed separately.

Table 4 Seasonal prevalence patterns in cattle and water buffaloes

Animal species	Season	Examined animals	Positive animals	Infection rate (% ± CI_{95})	P value
Cattle	Spring (Mar-Apr)	83	35	42.17 ± 10.80	0.05
	Summer (May-Aug)	164	70	42.68 ± 7.59	
	Autumn (Sep-Oct)	83	28	33.73 ± 10.35	
	Winter (Nov-Feb)	166	48	28.92 ± 7.04	
	Total	496	181	36.49 ± 4.28	
Water buffaloes	Spring (Mar-Apr)	80	25	31.25 ± 10.30	0.24
	Summer (May-Aug)	160	47	29.38 ± 7.15	
	Autumn (Sep-Oct)	80	20	25.00 ± 9.64	
	Winter (Nov-Feb)	156	36	23.08 ± 6.71	
	Total	476	128	26.89 ± 3.92	
All animals	Spring (Mar-Apr)	163	60	36.81 ± 7.54	0.03*
	Summer (May-Aug)	324	117	36.11 ± 5.27	
	Autumn (Sep-Oct)	163	48	29.45 ± 7.17	
	Winter (Nov-Feb)	322	84	26.09 ± 4.82	
	Total	972	309	31.79 ± 2.94	

Abbreviations: CI_{95} = 95% confidence interval; *P* value reported for χ^2 test; * = statistically significant ($p < 0.05$). Note: Seasons in Malakand Division were defined as: Winter (November-February), Spring (March-April), Summer (May-August), and Autumn (September-October).

3.5. Gender-based differences in gastrointestinal parasite prevalence

Gender-related differences in gastrointestinal parasite prevalence were observed, with contrasting patterns between cattle and water buffaloes (Table 5). In cattle, females exhibited a significantly higher prevalence (44.44%) compared to males (21.51%), representing a difference of 22.93 percentage points ($\chi^2 = 31.98$, $p < 0.01$). In contrast, water buffaloes showed an opposite pattern, with males displaying a higher prevalence (35.57%) than females (22.94%), corresponding to a difference of 12.63 percentage points ($\chi^2 = 8.88$, $p = 0.03$). When both species were combined, the overall prevalence remained higher in females (33.64%) than in males (28.04%); however, this difference was only marginally significant ($\chi^2 = 3.13$, $p = 0.05$). The contrasting sex-specific patterns observed between cattle and water buffaloes likely contributed to the attenuation of the overall gender effect in the combined analysis (Table 5).

Table 5 Gender-based differences in gastrointestinal parasite prevalence in cattle and water buffaloes

Animal species	Gender	Examined animals	Positive animals	Infection rate (% ± CI_{95})	P value
Cattle	Male	172	37	21.51 ± 3.11	0.00*
	Female	324	144	44.44 ± 2.77	
	Total	496	181	36.49 ± 4.28	
Water buffaloes	Male	149	53	35.57 ± 3.91	0.03*
	Female	327	75	22.94 ± 2.31	
	Total	476	128	26.89 ± 3.92	
All animals	Male	321	90	28.04 ± 2.51	0.05
	Female	651	219	33.64 ± 1.85	
	Total	972	309	31.79 ± 2.94	

Abbreviations: CI_{95} = 95% confidence interval; * = statistically significant ($p < 0.05$)

3.6. Age-dependent trends in gastrointestinal parasite prevalence

A clear age-dependent pattern in gastrointestinal parasite prevalence was observed in both cattle and water buffaloes (Table 6). In cattle, younger animals (<2 years) exhibited the highest prevalence (44.00%), followed by animals aged 2–7 years (39.89%), while the lowest prevalence

was observed in animals older than 7 years (30.05%). This decreasing trend with age was statistically significant (p = 0.02). A similar pattern was observed in water buffaloes, with the highest prevalence in animals younger than 2 years (37.78%), intermediate levels in the 2–7 years group (30.77%), and the lowest prevalence in animals older than 7 years (20.00%). This age-related difference was also statistically significant (p = 0.02). When both species were analyzed together, the age-dependent trend became more pronounced, with prevalence decreasing progressively from younger to older animals (41.05%, 35.69%, and 24.83%, respectively), and this difference was highly significant (p < 0.001) (Table 6).

Table 6 Age-dependent trends in gastrointestinal parasite prevalence in cattle and water buffaloes

Animal species	Age group	Examined animals	Positive animals	Infection rate (% ± CI_{95})	P value
Cattle	<2 years	100	44	44.00 ± 9.84	0.02*
	2–7 years	183	73	39.89 ± 7.15	
	>7 years	213	64	30.05 ± 6.14	
	Total	496	181	36.49 ± 4.28	
Water buffaloes	<2 years	90	34	37.78 ± 10.01	0.02*
	2–7 years	156	48	30.77 ± 7.31	
	>7 years	230	46	20.00 ± 5.19	
	Total	476	128	26.89 ± 3.92	
All animals	<2 years	190	78	41.05 ± 7.11	0.00*
	2–7 years	339	121	35.69 ± 5.15	
	>7 years	443	110	24.83 ± 4.05	
	Total	972	309	31.79 ± 2.94	

Abbreviations: CI_{95} = 95% confidence interval; * = statistically significant (p < 0.05).

3.8. Impact of drinking water source on gastrointestinal parasite prevalence

The source of drinking water showed variable associations with gastrointestinal parasite prevalence, with distinct patterns observed between cattle and water buffaloes (Table 7). In cattle, the highest prevalence was recorded in animals consuming municipal water (42.66%), followed by surface/rain/stored water (38.16%) and river water (37.31%), whereas the lowest prevalence was observed in animals using groundwater wells (19.61%). Although this represents a substantial difference of 23.05 percentage points, the variation was not statistically significant (p = 0.05). In water buffaloes, water source was significantly associated with parasite prevalence (p = 0.021). The highest infection rate was observed in animals consuming local stream water (41.03%), whereas the lowest prevalence occurred in those receiving municipal water (19.55%). Groundwater wells and surface/rain/stored water were associated with intermediate prevalence levels (20.00% and 27.05%, respectively). When both species were analyzed together, no significant association was detected between water source and parasite prevalence (p = 0.078), although higher prevalence was observed in animals using local stream water (36.65%) compared to those using groundwater wells (19.79%) (Table 7).

Table 7 Drinking water source on gastrointestinal parasite prevalence in cattle and water buffaloes

Animal species	Water source	Examined animals	Positive animals	Infection rate (% ± CI_{95})	P value
Cattle	Surface/rain/stored water	152	58	38.16 ± 7.92	0.05
	Groundwater well	51	10	19.61 ± 10.89	
	Local stream water	83	27	32.53 ± 10.26	
	River water	67	25	37.31 ± 11.76	
	Municipal water	143	61	42.66 ± 8.19	
	Total	496	181	36.49 ± 4.28	
Water buffaloes	Surface/rain/stored water	122	33	27.05 ± 7.96	0.02*
	Groundwater well	45	9	20.00 ± 11.81	
	Local stream water	78	32	41.03 ± 11.02	
	River water	98	28	28.57 ± 9.00	
	Municipal water	133	26	19.55 ± 6.89	
	Total	476	128	26.89 ± 3.92	
All animals	Surface/rain/stored water	274	91	33.21 ± 5.66	0.07
	Groundwater well	96	19	19.79 ± 8.00	
	Local stream water	161	59	36.65 ± 7.56	
	River water	165	53	32.12 ± 7.14	
	Municipal water	276	87	31.52 ± 5.46	
	Total	972	309	31.79 ± 2.94	

Abbreviations: CI_{95} = 95% confidence interval; * = statistically significant ($p < 0.05$).

3.9. Parasite egg counts and infection intensity

The intensity of gastrointestinal parasitism was assessed using eggs per gram (EPG) values and categorized infection levels (Tables 8 and 9). Substantial variation in parasite burden was observed among parasite taxa and between host species. Among all identified parasites, *H. contortus* exhibited the highest mean EPG values in both cattle (2000 ± 400) and water buffaloes (1600 ± 350), indicating a markedly higher parasite burden compared to other species (Table 8). Moderate EPG levels were observed for *O. ostertagi* and *Cooperia* spp., while relatively low EPG values were recorded for *Nematodirus* spp., *Toxocara* spp., and *Trichuris* spp. Overall, cattle tended to show higher EPG values than water buffaloes across most parasite species, suggesting a greater parasite burden in cattle. The distribution of infection intensity further supported these findings (Table 9). Across all infected animals, the majority were classified within the light (n = 122) and moderate (n = 90) infection categories, while fewer animals fell into the heavy (n = 66) and very heavy (n = 31) categories. A similar pattern was observed when cattle and water buffaloes were analyzed separately, indicating that although infection was common, severe parasite burdens were less frequent.

Table 8 Parasite species-specific egg counts (EPG)

Parasite	Cattle n	Mean EPG	Buffalo n	Mean EPG	All n	Mean EPG
Entamoeba spp.	21	450	13	380	34	423.2
Moniezia spp.	18	350	11	320	29	338.6
Haemonchus contortus	40	2000	32	1600	72	1822.2
Trichuris spp.	14	320	9	550	23	410.0
Ostertagia ostertagi	19	850	12	150	31	579.0
Cooperia spp.	20	650	14	550	34	608.8
Bunostomum phlebotomum	18	480	7	400	25	457.6
Nematodirus spp.	16	220	10	180	26	204.6
Toxocara spp.	17	180	12	150	29	167.6
Oesophagostomum spp.	23	380	8	320	31	364.5

Abbreviations: EPG = eggs per gram of feces; SD = standard deviation; n = number of animals.

Table 9 Classification of infection intensity in infected animals

Species	Light	Moderate	Heavy	Very heavy	Total
Cattle	72	54	38	17	181
Buffalo	50	36	28	14	128
All animals	122	90	66	31	309

Abbreviations: Light = 50-799; Moderate = 800-1199; Heavy = 1200-1999; Very heavy = >2000 EPG.

3.10. Molecular characterization and phylogenetic analysis of Haemonchus contortus

3.10.1. PCR amplification and sequence characteristics

Genomic DNA was successfully extracted from 35 adult *H. contortus* worms collected from the examined animals. PCR amplification of the ITS-1 region was successful for all 35 samples, yielding amplicons of approximately 238 base pairs, which is consistent with the expected fragment size for this marker. The obtained sequences were subjected to BLAST analysis against the NCBI GenBank database, confirming their identity as *H. contortus* with high sequence similarity. One representative sequence generated in this study (isolate DirPK05; accession number PV599769) was selected for downstream phylogenetic analysis based on sequence quality and alignment consistency.

3.10.2. BLAST sequence comparison of *Haemonchus contortus*

The obtained ITS/LSU rRNA sequence from the present study (isolate DirPK05; accession number PV599769) was subjected to BLAST analysis against sequences available in the NCBI GenBank database to confirm species identity and assess genetic similarity. BLAST results revealed highly significant alignments with previously reported *H. contortus* sequences (Table X). The query sequence showed 100% query coverage and 100% sequence identity with closely related isolates from Pakistan, including DirPK05 (PV599769.1), DirPK09 (PV621844.1), and DirPK10 (PV621845.1), with extremely low E-values (ranging from 8e-117 to 3e-111), indicating a very high level of sequence homology. Additionally, comparison with a geographically distant isolate from Uganda (Kab-16; accession number MW054171.1) revealed slightly lower query coverage (91%) and sequence identity (98.60%), suggesting minor genetic variation between regional populations while maintaining overall species-level conservation.

3.10.3. Phylogenetic relationships and evolutionary distance

Phylogenetic relationships among *H. contortus* isolates were inferred using the Neighbor-Joining method based on aligned ITS-1 sequences, with bootstrap analysis (≥1000 replicates) performed to assess the reliability of tree topology. The phylogenetic tree revealed the presence of two major clusters within the *H. contortus* species group, with a bootstrap support value of 83% for the node separating these clusters, indicating moderate to strong statistical support. Cluster 1 included the sequence generated in the present study (DirPK05; PV599769), which grouped closely with other Pakistani isolates (DirPK09 and DirPK10) and showed close genetic affinity with isolates from Uganda (Kab-16; MW054171) and additional reference sequences from diverse hosts, including sheep. This clustering suggests a high degree of genetic similarity among geographically distinct *H. contortus* populations. Cluster 2 comprised a larger group of isolates originating from multiple geographical regions, including Uganda, Indonesia, and India, and from different host species such as goats, sheep, and cervids. The broader diversity within this

cluster reflects the genetic variability of *H. contortus* across different ecological and host environments. Outgroup analysis was performed using *Oesophagostomum venulosum* (KF266764) and *Chabertia ovina* (KF266760), which formed distinct branches separate from the *H. contortus* clades, confirming the phylogenetic validity of the tree and the correct taxonomic placement of the studied sequences.

4. Discussion

The present study recorded an overall prevalence of gastrointestinal parasites of 31.79% among 972 cattle and water buffaloes in the Malakand Division, indicating a moderate level of infection. This finding is consistent with reports from other regions of Pakistan and South Asia, although higher prevalence rates have been documented elsewhere, such as 45.6% in Kohat [16] and 51% in cattle and 47% in buffaloes in southern Punjab [17]. These variations may reflect differences in climatic conditions, livestock management practices, and host species composition. Despite being moderate, the observed prevalence represents a significant threat to livestock productivity, particularly due to the predominance of *H. contortus*, a highly pathogenic parasite associated with anemia, reduced productivity, and mortality. These findings highlight the need for region-specific and evidence-based parasite control strategies in the Malakand Division.

Cattle exhibited a higher prevalence of gastrointestinal parasitism (36.49%) compared to water buffaloes (26.89%), suggesting differences in susceptibility and exposure between the two species. Similar patterns have been reported in Pakistan [18], indicating that this trend may be regionally consistent. This difference may be attributed to variations in grazing behavior, immune responses, and management practices. Cattle are more likely to graze extensively on contaminated pastures, increasing exposure to infective stages, whereas water buffaloes often engage in wallowing behavior, which may reduce contact with some parasites. Additionally, buffaloes may possess greater natural resistance due to long-term adaptation to local parasitic environments. These species-specific differences emphasize the importance of designing targeted control strategies that consider host-specific risk factors rather than applying uniform interventions across livestock species.

Despite sampling across nine districts within the Malakand Division, no statistically significant differences in gastrointestinal parasite prevalence were observed, with rates ranging from 24.14% to 41.67%. This relatively uniform distribution suggests that parasite transmission is influenced more by shared environmental and management conditions than by geographic location. Similar findings have been reported in other regions, including Lesotho, where no significant variation was observed across agro-ecological zones [19]. The lack of geographic differentiation in the present study may be attributed to comparable climatic conditions, similar husbandry practices, and frequent animal movement between districts, which can facilitate the spread of parasites and reduce spatial heterogeneity. Although higher prevalence was noted in certain districts such as Buner and Chitral Upper, these differences were not statistically significant and may reflect localized management or environmental factors rather than true geographic effects. Further studies incorporating detailed data on grazing systems, water sources, and animal movement patterns would be necessary to better understand these spatial dynamics.

Monthly analysis revealed notable fluctuations in gastrointestinal parasite prevalence throughout the study period (January-December 2022). In cattle, prevalence peaked in August (46.34%) and declined to its lowest level in December (19.51%), while water buffaloes showed a peak in March (35.00%) and a minimum in November (23.68%). These variations likely reflect the influence of climatic conditions, particularly temperature and humidity, on parasite development and survival. The higher prevalence observed during warmer and more humid months is consistent with previous findings in the same region, where peak *H. contortus* infections were reported during August [20]. Favorable environmental conditions during this period enhance the survival of infective stages on pasture and increase exposure during grazing. Despite these fluctuations, no statistically significant temporal trend was detected across months for cattle ($p > 0.05$), water buffaloes ($p > 0.05$), or the combined population ($p > 0.05$), suggesting that variability within months and between animals may obscure consistent linear patterns. Similar contrasts between visual trends and statistical significance have been reported in other studies [2], highlighting the complex and multifactorial nature of parasite transmission dynamics.

Seasonal analysis demonstrated a clearer pattern compared to monthly variation. When all animals were considered together, parasite prevalence was significantly higher during spring (36.81%) and summer (36.11%) than in autumn (29.45%) and winter (26.09%) ($p = 0.036$). This pattern is consistent with studies from similar climatic regions, such as Bangladesh [22], where higher parasite prevalence was observed during warmer and more humid seasons. The increased prevalence during spring and summer can be attributed to favorable environmental conditions for parasite development, increased grazing activity, and greater exposure to contaminated pastures. However, when analyzed separately, seasonal differences were not statistically significant for cattle ($p = 0.172$) or water buffaloes ($p = 0.249$), suggesting that species-specific factors and management practices may influence the strength of seasonal effects. This finding differs from previous reports in the region [23], which identified significant seasonal variation in both species. Such discrepancies may result from differences in sampling design, seasonal distribution of samples, or inter-annual environmental variability. Overall, these results indicate that while seasonal trends are biologically evident, their statistical expression may vary depending on host species and local conditions.

Marked gender-related differences in gastrointestinal parasite prevalence were observed, with contrasting patterns between cattle and water buffaloes. In cattle, females exhibited significantly higher infection rates (44.44%) compared to males (21.51%), indicating greater susceptibility in females ($p < 0.001$). This pattern is consistent with previous studies highlighting the role of sex hormones in modulating immune responses to parasitic infections [24,25]. Hormonal fluctuations associated with reproduction, particularly during pregnancy and lactation, may compromise

immunity and increase susceptibility [26]. In the present context, female cattle are primarily managed for dairy production, and the metabolic demands of reproduction likely contribute to their higher infection rates. In contrast, water buffaloes showed the opposite pattern, with males exhibiting higher prevalence (35.57%) than females (22.94%) ($p = 0.030$). This divergence suggests that factors beyond hormonal influences are involved. Behavioral and management-related differences, such as grazing patterns, habitat use, and exposure to contaminated environments, may play a key role. As reported by [27], gender-related variation in parasite burden can also be influenced by differences in feeding behavior, movement, and environmental exposure. These contrasting patterns highlight the importance of species-specific and gender-specific approaches to parasite control. Targeted interventions focusing on high-risk groups, as suggested by [28,29], may improve the effectiveness and cost-efficiency of control programs compared to uniform treatment strategies. Understanding these differences is essential for developing tailored management practices adapted to both host species and gender.

A clear inverse relationship between age and gastrointestinal parasite prevalence was observed in both cattle and water buffaloes, with younger animals (<2 years) consistently showing higher infection rates than older ones (>7 years). This trend was significant in both species and highly significant when all animals were combined, confirming age as a major risk factor. This pattern is widely reported and is primarily attributed to the gradual development of acquired immunity following repeated parasite exposure [30,31]. Younger animals lack this protective immunity, making them more susceptible to infection, whereas older animals develop partial resistance over time ("grazing immunity"). These findings have important practical implications, as age-targeted control strategies have been shown to improve parasite management efficiency [32,33]. Management practices, exposure patterns, and immune development all contribute to this age-related variation [34], highlighting the importance of prioritizing young animals in parasite control programs [35].

The association between drinking water source and parasite prevalence varied between species, indicating a complex interaction between environmental exposure and management practices. In cattle, although higher prevalence was observed in animals consuming municipal water and lower prevalence in those using groundwater, the association was not statistically significant ($p = 0.052$). This unexpected pattern may reflect confounding factors such as management conditions or infrastructure quality, as municipal water systems in some settings may still be vulnerable to contamination [36]. In contrast, water buffaloes showed a significant association ($p = 0.021$), with the highest prevalence in animals using local stream water and the lowest in those consuming municipal water. This aligns with the known role of surface water as a source of parasitic contamination [37], particularly given the frequent contact of buffaloes with aquatic environments. These findings suggest that water source influences parasitism differently across species, likely due to behavioral and management differences. Previous studies have similarly reported increased risk associated with surface water use [38,39]. Ensuring access to clean water and reducing exposure to contaminated sources should therefore be considered key components of parasite control strategies.

Haemonchus contortus was the dominant parasite in both cattle and water buffaloes, consistent with reports from South Asia [40], although regional variations exist [41]. Its predominance is likely linked to favorable climatic conditions and high reproductive capacity. Secondary parasites differed by species: *Oesophagostomum* spp. and *Cooperia* spp. were more common in cattle, while *Ostertagia ostertagi* and *Moniezia* spp. were more frequent in buffaloes, as also reported in other studies [42,43]. Less prevalent parasites included *Trichuris*, *Bunostomum*, and *Nematodirus*, which are typically minor components of parasite communities [44]. Differences between host species, including higher *Haemonchus* prevalence in buffaloes and higher *Oesophagostomum* in cattle, reflect host-specific exposure and management factors, consistent with previous findings [45].

Haemonchus contortus showed the highest EPG values, confirming its major role in parasite transmission, in agreement with previous studies [46,47], although regional differences have been reported [48]. In contrast, parasites such as *Trichuris* and *Oesophagostomum* exhibited lower EPG values, reflecting lower reproductive output [49]. Overall, EPG data indicate that while several parasites are present, *Haemonchus* is the primary contributor to environmental contamination and transmission risk.

The ITS-1 region proved to be a suitable marker for molecular characterization of *H. contortus*, as it combines conserved regions for amplification with sufficient variability for species discrimination, as previously reported [50]. BLAST analysis confirmed that the sequence obtained in this study showed very high similarity (up to 100%) with Pakistani isolates and strong similarity (≈98.60%) with isolates from Uganda, supporting accurate species identification. Slightly lower similarities with isolates from other regions (e.g., India and Indonesia) indicate normal intraspecific variation and suggest moderate geographic differentiation within *H. contortus* populations. Phylogenetic analysis using the Maximum Likelihood method revealed two main clusters within the *H. contortus* group, with moderate bootstrap support (83%). The sequence generated in this study clustered with Pakistani isolates and showed close relationships with isolates from different geographic regions, confirming both genetic conservation and limited geographic structuring. The use of *Oesophagostomum venulosum* and *Chabertia ovina* as outgroups clearly separated *H. contortus* from related species, supporting the robustness of the phylogenetic tree. These findings highlight the relatively conserved nature of *H. contortus* ITS-1 sequences while indicating some level of regional genetic variation. Molecular characterization provides a useful baseline for future studies on parasite diversity and potential anthelmintic resistance monitoring, particularly in regions where phenotypic testing is limited.

The study has some limitations. The cross-sectional design does not allow causal inference, and the limited number of sequenced isolates ($n = 35$) may not fully capture genetic diversity. In addition, incomplete data on management practices may have influenced observed epidemiological patterns. From a practical perspective, the results support targeted parasite control strategies in the Malakand Division. Priority should be given

to young animals, high-risk groups, and control of *Haemonchus* spp., particularly during high-transmission seasons. Integrated approaches combining improved management, strategic treatment, and monitoring are essential for effective long-term control.

5. Conclusion

This study provides a comprehensive assessment of gastrointestinal parasitism in cattle and water buffaloes in the Malakand Division, revealing a moderate overall prevalence (31.79%) with significant variation across host species, age groups, gender, season, and management factors. H. contortus was identified as the dominant parasite, both in prevalence and infection intensity, highlighting its major role in parasite transmission and associated production losses. Younger animals and specific host groups (female cattle and male buffaloes) were found to be at higher risk, while seasonal and environmental factors further influenced infection patterns. Molecular characterization confirmed the identity of H. contortus and demonstrated high genetic similarity with global isolates, with moderate regional variation. These findings emphasize the need for integrated, targeted parasite control strategies focusing on high-risk groups, improved management practices, and continuous monitoring, including molecular surveillance, to enhance livestock productivity and sustainability in the region.

Declarations

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Author Contributions

Tawseef Khan: Methodology, Investigation, Data Curation, Formal Analysis, Writing – Original Draft Preparation. Nasreen Nasreen: Conceptualization, Methodology, Validation, Data Curation, Formal Analysis, Writing – Original Draft Preparation. Sadaf Niaz: Data Curation, Investigation, Formal Analysis. Adil Khan: Methodology, Validation, Data Analysis. Ayman A Swelum: Formal Analysis, Writing – Review & Editing. Ruhoollah: Investigation, Data Collection. Wajid Ali: Validation, Writing – Review & Editing. Mourad Ben Said: Data Curation, Formal Analysis, Validation, Data Analysis, Writing – Original Draft Preparation, Writing – Review & Editing.

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All data generated or analyzed during this study are included in this published article.

Code Availability

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Hashmi HA, Belgacem AO, Behnassi M, Javed K, Baig MB (2021) Impacts of climate change on livestock and related food security implications—overview of the situation in Pakistan and policy recommendations. In: Emerging challenges to food production and security in Asia, Middle East, and Africa. Springer, pp 197–239. https://doi.org/10.1007/978-3-030-72987-5_8
2. https://www.pbs.gov.pk/publication/pakistan-mouza-census-2020-country-report
3. Rehman A, Jingdong L, Chandio AA, Hussain I (2017) Livestock production and population census in Pakistan: determining their relationship with agricultural GDP using econometric analysis. Inf Process Agric 4(2):168–177. https://doi.org/10.1016/j.inpa.2017.03.002
4. Heema P (2023) Prevalence of gastrointestinal parasitic infections and haemoprotozoan diseases of buffalo in coastal areas of Chattogram division, Bangladesh. Doctoral dissertation, Chattogram Veterinary and Animal Sciences University, Chattogram
5. Verocai, G. G., Chaudhry, U. N., & Lejeune, M. (2020). Diagnostic Methods for Detecting Internal Parasites of Livestock. *The Veterinary clinics of North America. Food animal practice*, *36*(1), 125–143. https://doi.org/10.1016/j.cvfa.2019.12.003
6. Strydom T, Lavan RP, Torres S, Heaney K, 2023. The Economic Impact of Parasitism from Nematodes, Trematodes and Ticks on Beef Cattle Production. Animals (Basel). 10;13(10):1599. https://doi.org/10.3390/ani13101599

7. Vande Velde F, Charlier J, Claerebout E. 2018. Farmer Behavior and Gastrointestinal Nematodes in Ruminant Livestock-Uptake of Sustainable Control Approaches. Front Vet Sci. Oct 16;5:255. https://doi.org/10.3389/fvets.2018.00255
8. Hamid L, Alsayari A, Tak H, Mir SA, Almoyad MAA, Wahab S, Bader GN (2023) An insight into the global problem of gastrointestinal helminth infections amongst livestock: does nanotechnology provide an alternative? Agriculture 13:1359. https://doi.org/10.3390/agriculture13071359
9. Khan R, Al-Jabr OA, Khan T, Khan A, El-Ghareeb WR, Aguilar-Marcelino L (2023) Prevalence of gastrointestinal parasite in small ruminants of District Dir Upper Khyber Pakhtunkhwa Province of Pakistan. Braz J Biol 83:e248978. https://doi.org/10.1590/1519-6984.248978
10. Irshad A, Noreen S, Khan W, Rasool A (2024) Sero-epidemiology, spatial distribution and phylogenetic analysis of *Toxoplasma gondii* in goats of Malakand Division of Pakistan. Pak Vet J. https://doi.org/10.29261/pakvetj/2024.152
11. van Dijk J, Sargison ND, Kenyon F, Skuce PJ (2010) Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. Animal 4(3):377–392. https://doi.org/10.1017/S1751731109990991
12. Squire SA, Robertson ID, Yang R, Ayi I, Ryan U (2019) Prevalence and risk factors associated with gastrointestinal parasites in ruminant livestock in the coastal savannah zone of Ghana. Acta Trop 199:105126. https://doi.org/10.1016/j.actatropica.2019.105126
13. Adanir R, Tasci F (2013) Prevalence of helminth eggs in raw vegetables consumed in Burdur, Turkey. Food Control 31(2):482–484. https://doi.org/10.1016/j.foodcont.2012.10.032
14. Foreyt WJ. Diagnostic parasitology. In: Veterinary parasitology reference manual. 5th ed. Ames (IA): Iowa State University Press; 2001. p. 3-10.
15. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023
16. Rashid A, Khattak MNK, Khan MF, Ayaz S, Rehman AU (2016) Gastrointestinal helminthoses: prevalence and associated risk factors in small ruminants of District Kohat, Pakistan. J Anim Plant Sci 26(4):956–962
17. Raza MA, Iqbal Z, Jabbar A, Yaseen M (2007) Point prevalence of gastrointestinal helminthiasis in ruminants in southern Punjab, Pakistan. J Helminthol 81(3):323–328. https://doi.org/10.1017/S0022149X07818554
18. Khan MN, Sajid MS, Khan MK, Iqbal Z, Hussain A (2010) Gastrointestinal helminthiasis: prevalence and associated determinants in domestic ruminants of District Toba Tek Singh, Punjab, Pakistan. Parasitol Res 107(4):787–794. https://doi.org/10.1007/s00436-010-1931-x
19. Matsepe LG, Molapo S, Phalatsi M, Phororo M (2021) Prevalence and fecal egg load of gastrointestinal parasites of Angora goats in four agro-ecological zones in Lesotho. Vet World 14(2):339–346. https://doi.org/10.14202/vetworld.2021.339-346
20. Rashid S, Irshadullah M (2018) Epidemiology and seasonal dynamics of adult *Haemonchus contortus* in goats of Aligarh, Uttar Pradesh, India. Small Rumin Res 161:63–67. https://doi.org/10.1016/j.smallrumres.2018.01.018
21. Mohamed HI, Arafa WM, El-Dakhly KM (2023) Prevalence and associated risk factors of gastrointestinal helminths and coccidian infections in domestic goats (*Capra hircus*) in Minya, Egypt. Beni-Suef Univ J Basic Appl Sci 12:29. https://doi.org/10.1186/s43088-023-00369-6
22. Amran MA, Yadav SK, Akter F, Sarkar S, Hossain MA, Joy SM, Samrat AAK (2018) Prevalence of gastrointestinal parasitic infections in different existing goat breeds in different districts of Bangladesh. J Adv Parasitol 5(1):11–21. https://doi.org/10.17582/journal.jap/2018/5.1.11.21
23. Shit N, Hajra DK, Baidya S, Debbarma A (2017) Seasonal occurrence of gastrointestinal helminth parasites in cattle and buffaloes in Bankura District, West Bengal, India. Explor Anim Med Res 7(1):58–63
24. Roberts CW, Walker W, Alexander J (2001) Sex-associated hormones and immunity to protozoan parasites. Clin Microbiol Rev 14(3):476–488. https://doi.org/10.1128/CMR.14.3.476-488.2001
25. Klein SL (2004) Hormonal and immunological mechanisms mediating sex differences in parasite infection. Parasite Immunol 26(6–7):247–264. https://doi.org/10.1111/j.0141-9838.2004.00710.x
26. Fair T (2015) The contribution of the maternal immune system to the establishment of pregnancy in cattle. Front Immunol 6:7. https://doi.org/10.3389/fimmu.2015.00007
27. Mochankana ME, Robertson ID (2018) Cross-sectional prevalence of *Fasciola gigantica* infections in beef cattle in Botswana. Trop Anim Health Prod 50(6):1355–1363. https://doi.org/10.1007/s11250-018-1568-9
28. Khan T, Khan W, Iqbal R, Maqbool A, Fadladdin YAJ, Sabtain T (2022) Prevalence of gastrointestinal parasitic infection in cows and buffaloes in Lower Dir, Khyber Pakhtunkhwa, Pakistan. Braz J Biol 83:e242677. https://doi.org/10.1590/1519-6984.242677
29. Gurung K, Tulachan PM, Gauchan D (2005) Gender and social dynamics in livestock management: a case study from three ecological zones in Nepal. Centre for Mountain Research Development (CEMORD) and National Agricultural Research Centre, Kathmandu
30. Hoar BR, Atwill ER, Elmi C, Farver TB (2001) An examination of risk factors associated with beef cattle shedding pathogens of potential zoonotic concern. Epidemiol Infect 127(1):147–155. https://doi.org/10.1017/S0950268801005726
31. Roberts FHS, O'Sullivan PJ, Riek RF (1952) The epidemiology of parasitic gastro-enteritis of cattle. Aust J Agric Res 3(2):187–226

32. Leathwick DM, Sauermann CW, Nielsen MK (2019) Managing anthelmintic resistance in cyathostomin parasites: investigating the benefits of refugia-based strategies. Int J Parasitol Drugs Drug Resist 10:118–124. https://doi.org/10.1016/j.ijpddr.2019.08.008
33. Charlier J, Morgan ER, Rinaldi L, van Dijk J, Demeler J, Höglund J, Hertzberg H, Van Ranst B, Hendrickx G, Vercruysse J, Kenyon F (2014) Practices to optimise gastrointestinal nematode control on sheep, goat and cattle farms in Europe using targeted (selective) treatments. Vet Rec 175(10):250–255. https://doi.org/10.1136/vr.102512
34. Wiersma E, Pakeman RJ, Bal X, Pilkington JG, Pemberton JM, Nussey DH, Sweeny AR (2023) Age-specific impacts of vegetation functional traits on gastrointestinal nematode parasite burdens in a large herbivore. J Anim Ecol 92(9):1869–1880. https://doi.org/10.1111/1365-2656.13978
35. Murugan R, Kumar B, Patel GM (2023) Systematic review of oestrosis in small livestock: understanding the impact and management strategies. Red Vet 24(4)
36. Karanis P, Kourenti C, Smith H (2007) Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health 5(1):1–38. https://doi.org/10.2166/wh.2006.002
37. Geldreich EE (1996) Pathogenic agents in freshwater resources. Hydrol Process 10(2):315–333
38. Daniels ME, Shrivastava A, Smith WA, Sahu P, Odagiri M, Misra PR, Panigrahi P, Suar M, Clasen T, Jenkins MW (2015) Cryptosporidium and Giardia in humans, domestic animals, and village water sources in rural India. Am J Trop Med Hyg 93(3):596–600. https://doi.org/10.4269/ajtmh.15-0111
39. Nurhidayah N, Satrija F, Retnani EB, Astuti DA, Murtini S (2019) Prevalence and risk factors of trematode infection in swamp buffaloes under different agro-climatic conditions in Java, Indonesia. Vet World 13(4):687–694. https://doi.org/10.14202/vetworld.2020.687-694
40. Nath S, Dixit AK, Jain R, Agrawal V, Dongre S, Das G (2015) Epizootiological studies on gastrointestinal parasites of domestic ruminants in Khandwa District of Madhya Pradesh, India. J Vet Parasitol 29(1):1–9
41. Farhood NN, Al-Idreesi SR (2020) Identification of *Strongyloides papillosus* and other gastrointestinal parasites of cattle in Basrah Province. Bas J Vet Res 19(1):155–168. https://doi.org/10.23975/bjvetr.2020.170620
42. Mamatha GS, D'Souza PE (2006) Gastro-intestinal parasitism of cattle and buffaloes in and around Bangalore. J Vet Parasitol 20:163–165
43. Raza MA, Bachaya HA, Akhtar MS, Arshad HM, Murtaza S, Ayaz MM, Basit A (2012) Point prevalence of gastrointestinal helminthiasis in buffaloes (*Bubalus bubalis*) at Jatoi, Punjab, Pakistan. Sci Int (Lahore) 24(4):465–469
44. Krishnamoorthy P, Lakshmi HK, Jacob SS, Suresh KP, Patil SS (2024) Gastrointestinal parasites in dairy cattle and buffaloes across climatic regions: a scientometric approach. Vet Parasitol Reg Stud Reports 47:100966. https://doi.org/10.1016/j.vprsr.2023.100966
45. Abbas M, Kumar L, Tunio S, Soomro AG, Farooq MM, Yousaf A, Lanjar Z (2021) Prevalence of gastrointestinal parasites in buffalo and cow calves in rural areas of Rawalpindi, Pakistan. Biomed J Sci Tech Res 40(2): 32159-2165
46. Yadav CL, Kumar RR, Vatsya S, Garg R, Baneerjee PS (2008) Epidemiological studies on gastrointestinal nematodosis in cattle and buffaloes. J Vet Parasitol 22(1):49–52
47. Rahman H, Pal P, Bandyopadhyay S, Chatlod LR (2012) Epidemiology of gastrointestinal parasitism in cattle in Sikkim. Indian J Anim Sci 82(2)
48. Jithendran KP, Bhat TK (1999) Epidemiology of parasitoses in dairy animals in the North West Himalayan region with emphasis on gastrointestinal nematodes. Trop Anim Health Prod 31(4):205–214. https://doi.org/10.1023/A:1005263009921
49. Ojeda-Robertos NF, Torres-Acosta JFJ, González-Garduño R, Notter DR (2017) Phenotypic expression of susceptibility to *Haemonchus contortus* in Pelibuey sheep. Vet Parasitol 239:57–61. https://doi.org/10.1016/j.vetpar.2017.04.015
50. Yao H, Song J, Liu C, Luo K, Han J, Li Y, Pang X, Xu H, Zhu Y, Xiao P, Chen S (2010) Use of ITS2 region as a universal DNA barcode for plants and animals. PLoS One 5(10):e13102. https://doi.org/10.1371/journal.pone.0013102

Figures

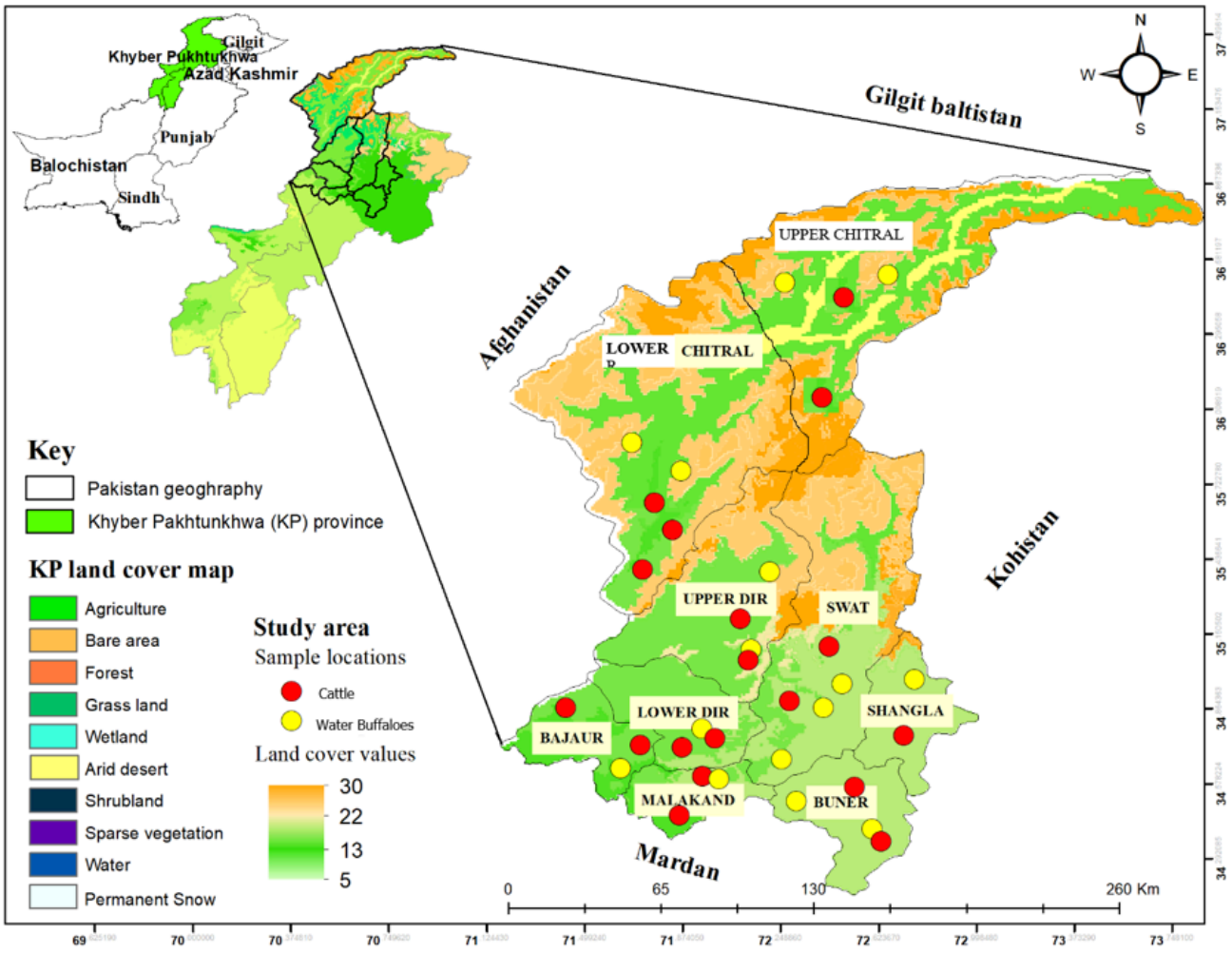

Figure 1

Map of study area (Malakand Division of Pakistan)

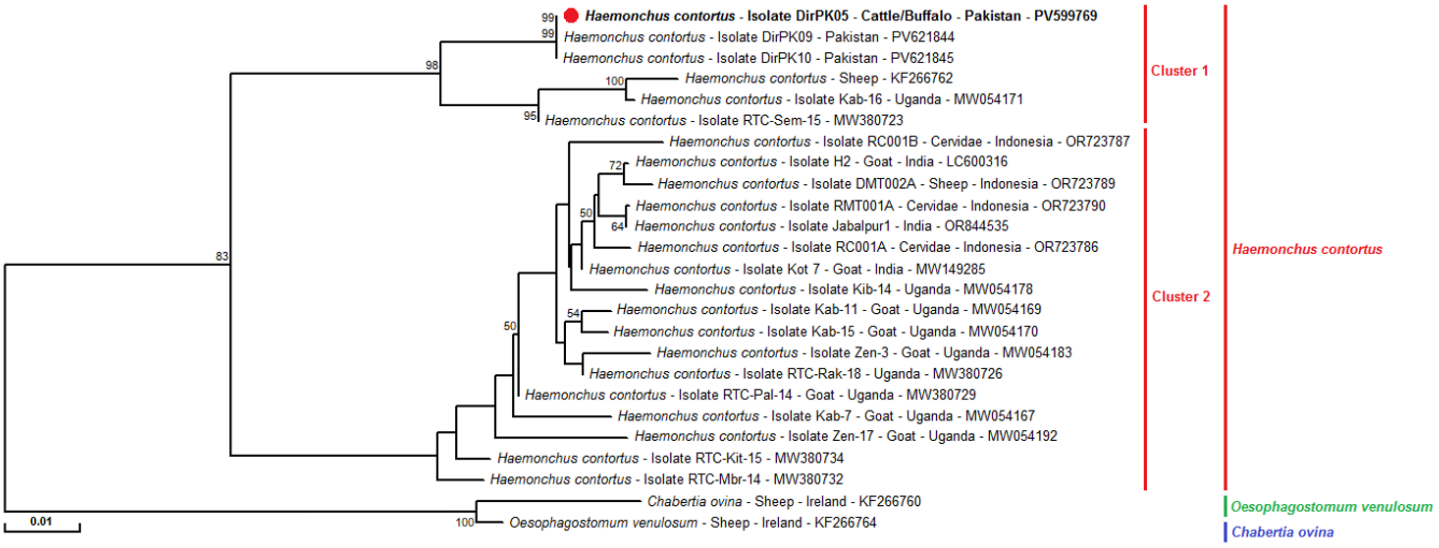

Figure 2

Phylogenetic tree of *Haemonchus contortus* ITS-1 sequences based on Maximum Likelihood (ML) analysis

Legend: The phylogenetic tree was constructed using aligned ITS-1 sequences of *H. contortus* isolates obtained from the present study and reference sequences retrieved from GenBank. Bootstrap values (≥1000 replicates) are indicated at the nodes. The sequence generated in this study (isolate DirPK05; accession number PV599769) is highlighted in bold and marked with a red circle. Two closely related nematode species, *Oesophagostomum venulosum*(KF266764) and *Chabertia ovina* (KF266760), were used as outgroups to root the tree.