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PREVALENCE MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF TICK SPECIES: A POTENTIAL VECTOR FOR CRIMEAN CONGO HEMORRHAGIC FEVER (CCHF)

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ABSTRACT

The objective of this study was to identify the prevalent tick species infesting different livestock animals in Pakistan. Ticks are obligate hematophagous arthropods that are widely distributed across the globe and are second only to mosquitoes in transmitting life-threatening infectious diseases. They serve as vectors of numerous viral, bacterial, and protozoal pathogens, including Crimean Congo Hemorrhagic Fever (CCHF). A total of 389 tick specimens were collected, and the geographic coordinates of each sampling location were recorded using a hand-held Global Positioning System (GPSMAP Garmin 62S). Morphological identification was performed under stereo and electron microscopes using standard taxonomic keys, and specimens were classified according to genus, species, and sex.Out of the total 389 ticks examined, Hyalomma excavatum (n=110), Hyalomma dromedarii (n=169), Rhipicephalus decoloratus (n=105), Rhipicephalus microplus (n=2), and Rhipicephalus appendiculatus (n=3) were identified. The prevalence of each species was as: H. appendiculatus 0.77%, R. decoloratus 27%, R. microplus 0.5%, H. dromedarii 43%, and H. excavatum 28%. Among these, H. dromedarii and H. excavatum were the most dominant species infesting livestock in the study areas. Given that these species are recognized as competent vectors for CCHF, their high prevalence combined with the reported cases of CCHF in urban areas of Pakistan underscores a significant public health risk. This risk may be amplified during Eid-ul-Adha due to increased human animal contact. This study may support in strategies to control the spreading of disease to human population and particularly by adopting integrated approaches for the control of vectors in affected areas of Pakistan.

Keywords: *Crimean congo hemorrhagic fever, Livestock, Ticks*

INTRODUCTION

Ticks are obligate hematophagous ectoparasites belonging to the phylum *Arthropoda*. They are widely distributed across diverse ecological regions and are recognized as vectors of numerous bacterial, protozoal, and viral pathogens responsible for severe and often fatal diseases, including tick-borne encephalitis, Mediterranean spotted fever, Crimean-Congo hemorrhagic fever (CCHF), and Siberian tick typhus (1). In addition to transmitting infectious agents, tick infestations negatively impact livestock health by reducing productivity and causing significant economic losses. Ticks have adapted their feeding behavior to parasitize nearly all terrestrial mammals, birds, and reptiles (2). Globally, approximately 900 tick species have been described, of which nearly 700 belong to the family Ixodidae (hard ticks) and about 200 to the family Argasidae (soft ticks). After mosquitoes, ticks are considered the second most important arthropod vector of human and animal diseases (3). Moreover, large-scale analyses based on morphological traits and molecular markers of different tick species provide valuable insights into their phylogeny and evolutionary relationships (7).

In Pakistan, CCHF is endemic, with human cases increasing over the past decade. Recent surveillance has confirmed circulating virus in both livestock and ticks, and genotype IV (Asia-1) is predominant (13). For example, a study in Balochistan reported approximately 4% of *Hyalomma* ticks positive for CCHFV genomes, with *H. marginatum*, *H. excavatum*, *H. dromedarii*, *H. anatolicum*, and *H. scupense*

among the dominant species (14). Tick ecology in Pakistan is complex and influenced by climatic, geographic, and host factors. Several studies have mapped species distribution, showing *Hyalomma anatolicum* and *Rhipicephalus microplus* as among the most frequently encountered species on livestock. Geographic epidemiology indicates that hot-spot areas for these ticks (and tick-borne diseases) include northwestern and northcentral Pakistan (15).

Recent molecular studies have refined species identification and revealed novel records. For example, Obaid *et al.*, (2024) provided the first molecular confirmation of *Hyalomma asiaticum* in Pakistan, using cytochrome oxidase subunit I (cox1) gene sequencing, alongside detection of Rickettsiales bacteria in various tick species (16). Another study in Sindh province used molecular tools to validate morphologically identified *Hyalomma* species and also tested acaricidal susceptibility (17). Despite these advances, gaps remain. There is limited recent data on the prevalence, distribution, and molecular profiles of less common *Hyalomma* species in certain provinces, and their direct association with CCHFV transmission in livestock are under-studied.

MATERIALS AND METHODS

FIELD SAMPLING AND ETHICAL CONSENT

Verbal consent was obtained from the owners of all farm animals prior to sampling. Tick specimens were collected between January 2018 and January 2019. Sampling was performed using sterile forceps, with strict adherence to biosafety precautions, including the use of gloves and face masks, particularly when handling engorged ticks at different life stages (larvae, nymphs, and adults).

SAMPLE PRESERVATION/STORAGE

Samples were collected in sealed containers containing tick preservative solution (ethanol 79%, distilled water 15%, glycerol 5%, and chloroform 1%) for transportation (4).

ENVIRONMENTAL PARAMETERS

Temperature and relative humidity at sampling sites were recorded using standardized data forms. During the collection period, the temperature ranged from 32°C to 40°C and relative humidity ranged from 32% to 57%.

GEOGRAPHIC INFORMATION SYSTEM (GIS) MAPPING

The latitude and longitude of sampling locations in Karachi city were recorded using a hand-held Global Positioning System (GPSMAP Garmin 62S) (Fig. 1). This information is valuable for analyzing spatial and temporal patterns using geospatial mapping. Spatial distribution helps identify potential breeding sites of tick vectors, while temporal trends can reveal and explain fluctuations in tick populations over time.

LAB WORK

Morphological Identification: All samples were studied and morphologically identified with the help of keys given in "Ticks of Domestic Animal in Africa: A guide to Identification of Species by (11) Stereo and Electron Microscope: Ticks identification was carried out under the stereo and electron microscope (Fig. 2).

Following morphological identification, most samples were pooled according to species, sex, and location, and stored at –80°C for subsequent CCHF virus screening by RT-PCR. Few representative individual specimens were preserved for species confirmation by molecular methods.

MOLECULAR IDENTIFICATION OF TICK SPECIES

All pre-inactivation procedures were performed in a Class II biosafety cabinet within ABSL-3 containment facilities. Tick specimens were placed in 2 mL OMNI bead rupture tubes, washed twice with sterile phosphate-buffered saline (PBS, pH 7.4), and homogenized using pre-sterilized stainless-steel beads (2.4 mm). Homogenization was carried out in an OMNI Bead Ruptor equipped with a cryo-cooling unit using the following settings: speed 6, 10 cycles, 10 s run time with 15 s intervals. Samples were cooled on ice

between cycles and subsequently centrifuged at 4,000–5,000 rpm for 10–12 min. Approximately 250 µL of the clarified supernatant was collected for DNA extraction using a commercial kit (GeneJET Genomic DNA Extraction Kit, K0721) according to the manufacturer's instructions. All consumables and waste were decontaminated using 5% sodium hypochlorite prior to disposal (8, 9).

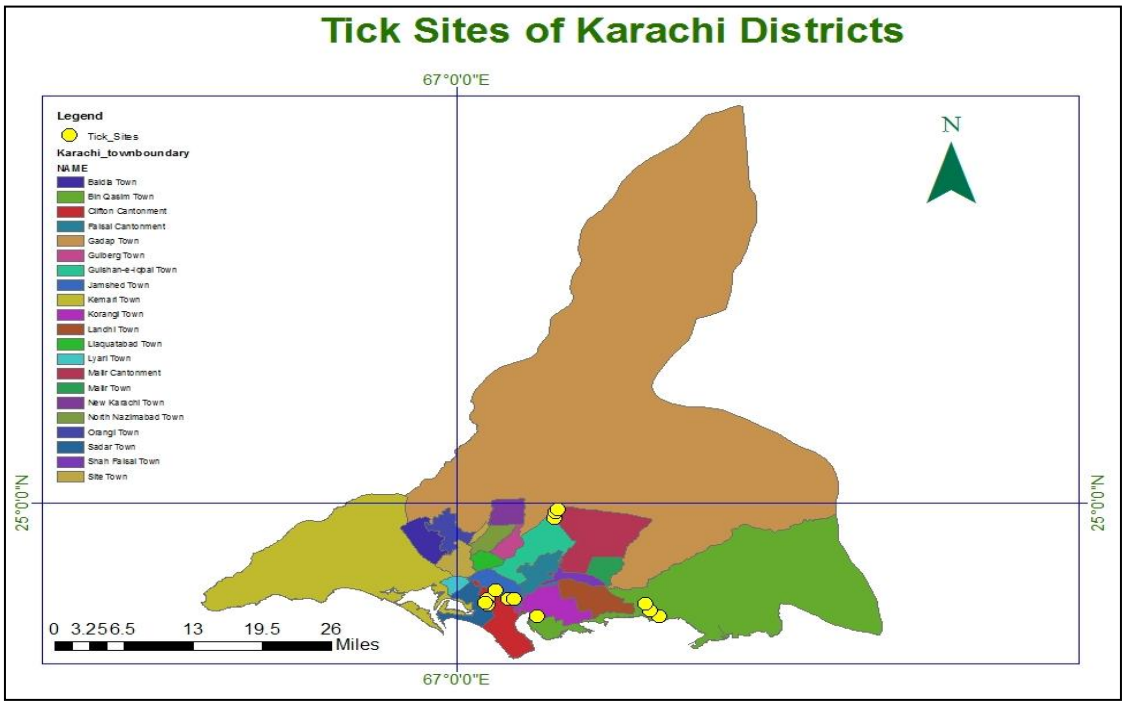

Fig. 1. GPS surveillance spot

PCR amplification of the second internal transcribed spacer (ITS2) gene was performed using Hot Start Green PCR Master Mix (2×). Each 25 µL reaction contained 12.5 µL master mix, 1 µL each of ITS-F and ITS-R primers (10 pmol/µL), 2 µL template DNA, and 8.5 µL nuclease-free water. Reaction mixtures were gently vortexed and briefly centrifuged prior to amplification (Table I).

Table I. Sequence of primers for PCR amplification and their resultant PCR product size

Primer (Gene amplification)	Sequence (5/-3/)	Nature	Expected product size (bp)
ITS-F	AGGACA TGA GCA ATTC	Universal	~750
ITS-R	ACT GCG AAG CAC TTR GAC CG		(3)

The template Control was prepared by mixing 2X Master Mix, Forward and Reverse Primers, and Nuclease Free Water (total volume 25 µL in quantities mentioned except quantity of nuclease-free water to be added is 10.5 µL). The samples were gently vortexed and PCR was performed using the recommended thermal cycling conditions (12).

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed using a 1.5% agarose gel prepared in 1× TAE buffer containing ethidium bromide (0.5 µg/mL). PCR products mixed with loading dye (5–10 µL) were loaded into the gel wells, and a 100 bp DNA ladder was used as a molecular size marker. Electrophoresis was carried out in 1× TAE buffer at 5 V/cm for 60 minutes. Amplified DNA fragments were visualized using a gel documentation system, and product sizes were determined by comparison with the DNA ladder (Fig. 2).

PURIFICATION OF AMPLIFIED DNA PRODUCT

The desired amplified DNA fragments were purified from agarose gels using the Thermo Scientific GeneJET Gel Extraction Kit (Cat. No. K0691), following the manufacturer's instructions.

SEQUENCING & BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST) ANALYSIS

Purified DNA samples were submitted for commercial sequencing. Raw sequence data were assembled using appropriate bioinformatics software (e.g., SeqMan). Tick species were confirmed by

performing BLAST analysis against the NCBI nucleotide database (www.ncbi.nlm.nih.gov/blast/). Sequence alignments were conducted using programs such as Jalview or MEGA, and phylogenetic trees were constructed, when required, to infer evolutionary relationships among species (10).

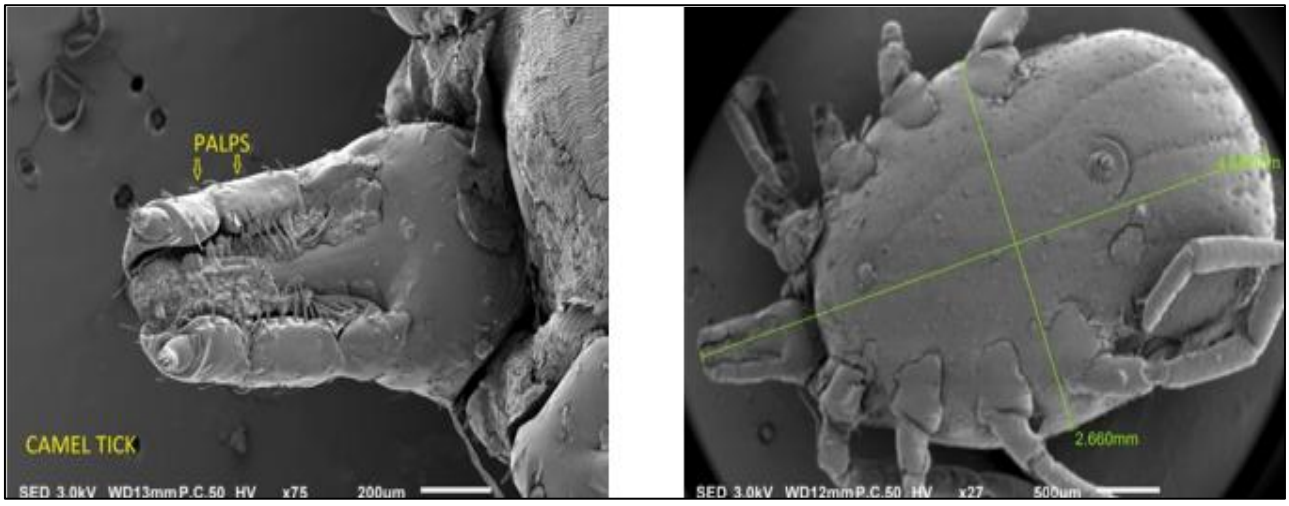

Fig. 2. Microscopic examination of tick parts; **(a).** Electron microscopic image of tick mouthpart; **(b).** Electron microscopic image of tick Diaphragm

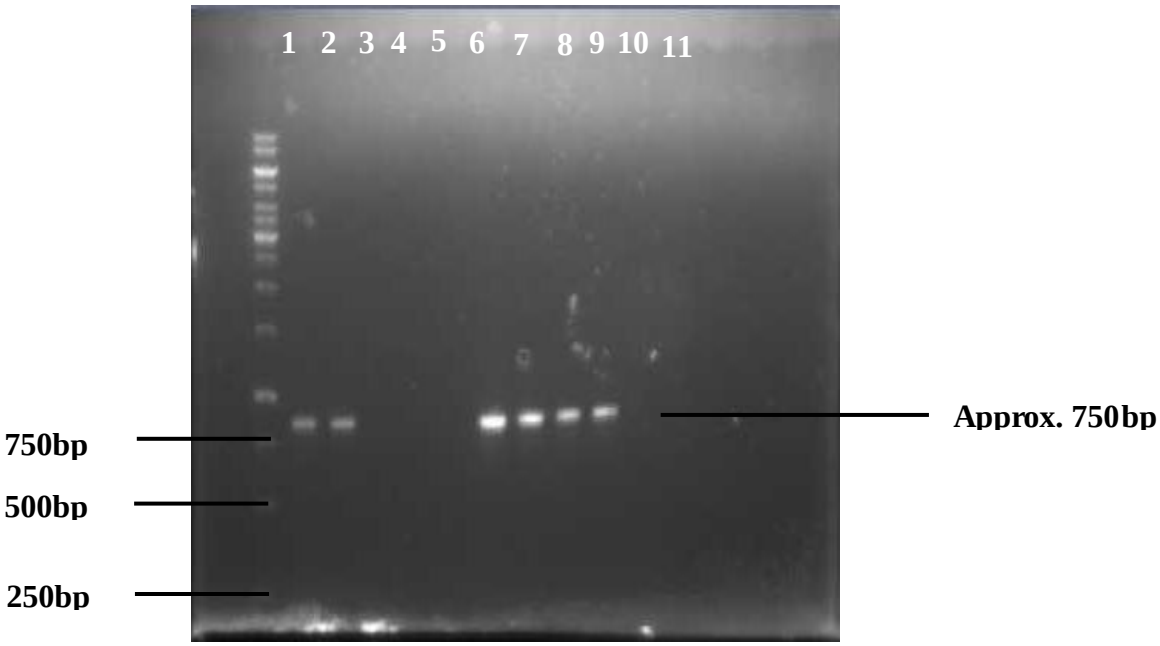

Fig. 3. Tick (750bp) PCR product: Internal Transcribed Spacer (ITS-2) Genes
Key: Lane 1:1kb DNA ladder (Thermoscientific, SM0313); Lane 2, 3, 7 to 10: PCR Product Tick (750bp); Lane 11: Negative Control

STATISTICAL ANALYSIS

SPSS was used for descriptive statistics to calculate the prevalence (%) of tick species across the sampling areas. Microsoft Excel was also used for visual interpretation to determine the prevalence of each species in specific areas. A Chi-square test was applied to determine the prevalence percentages of tick species across different hosts.

RESULTS

Out of total 389 ticks, 110 were identified as a *Hyalomma aexcavatum* (42, 11, 13 and 44 from bull, cow, camel and goat respectively), 169 as *Hylomma dromedarii* (133, 3, 20 and 13 from camel, cow, bull and goat respectively), 105 as *Rhipicephalus decoloratus* (103 and 2 from cow and goat respectively), 2 as *Rhipicephalus microplus* (Camel) and 3 as *Rhipicephalus appendiculatus* (goat) (Table II). *Hyalomma dromadarri*, 43%, and *Hyalomma Excavatum*, 28% were found to be the most prevalent ticks species infesting the livestock of the sample's sites (Table III).

Table II. Total number of tick species in different counties of Quetta and Karachi

No	Sampling date	Area	Host	Genus/Specie	Total
1	19-04-2018	Bance Colony	Cow	Rhipicephalusdecoloratus	103
2	23-04-2018	Qayyum Abad	Camel	Hyalommadromedarii	5
3	13-08-2018	Maveshi Mandi Super High Way	Bull	Hyalommaexcavatum	15
			Cow		7
4	15-08-2018	Camel Mandi (Islam Coat)	Camel	Hyalommadromedarii	17
				Hyalommaexcavatum	13
				Rhipicephalusmicroplus	2
5	16-08-2018	Kala Pull	Cow	Hyalommaexcavatum	4
				Hyalommadromedarii	2
6	17-08-2018	MaveshiMandi Super High way	Goat	Hyalommaexcavatum	3
			Cow	Hyalommadromedarii	1
7	18-08-2018	MaveshiMandi Race Course	Bull	Hyalommaexcavatum	27
				Hyalommadromedarii	20
			Goat	Hyalommaexcavatum	17
				Hyalommadromedarii	13
				Rhipicephals Appendiculatus	3
8	10/12/2018	Quetta	Camel	Hyalommadromedarii	111
9	28-01-2019	Akhtar Colony	Goat	Hyalommaexcavatum	24
				Rhipicephalusdecoloratus	2
		Total			389

Table III. Percentage (%) of tick species in different counties of Quetta and Karachi

Localities	*H. excavatum*	*H. dromedarii*	*H. appendiculatus*	*R. decoloratus*	*R. microplus*
Akhtar Colony	_	5	_	103	_
Quetta	_	_	_	_	_
Maveshi Mandi Race Course	22	_	_	_	_
Maveshi Mandi Super Highway	13	17	_	_	2
Kala Pull	4	2	_	_	_
Camel Mandi Islam Coat	3	1	_	_	_
Maveshi Mandi Super Highway	44	33	3	_	_
Qayyum Abad	_	111	_	_	_
Bans Colony	24	_	_	2	_
Total	110	169	3	105	2
Percentage	28%	43%	0.77%	27%	0.5%

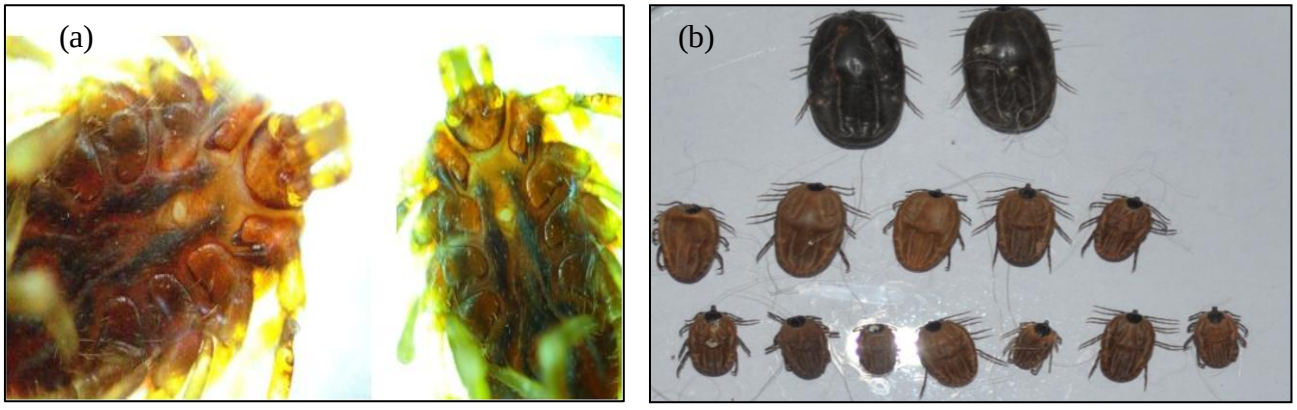

Fig. 3 (a). Florescence microscope images of Hylomma ticks; **(b).** Microscopic images of Hylomma ticks

MORPHOLOGICAL IDENTIFICATION OF SPECIES

The analysis was performed using Microsoft Excel, and a chart was generated to illustrate the distribution of morphologically identified tick species across different hosts and sampling area as shown in

Fig. 4. Among the identified tick species, *Hyalomma dromedarii* and *Hyalomma excavatum* were the most prevalent, infesting all host animals examined (cows, camels, bulls, and goats) and distributed across all sampling sites except Bhains Colony. In contrast, *Hyalomma decoloratus* showed a marked prevalence in cattle from Bhains Colony but was absent in goats from Akhtar Colony. *Rhipicephalus microplus* was rarely detected and occurred only in camels sampled at Camel Mandi (Islam Coat). Similarly, *Rhipicephalus appendiculatus* was identified at very low frequency, restricted to goats in the Maveshi Mandi race course area (Fig. 4).

PREVALENT SPECIES AMONG AREAS

Descriptive statistics were used to calculate the prevalence (%) of tick species across the sampling areas as shown in Table IV and graphical representation of prevalent species among areas shown in Fig. 5. The percentages of the species show that *Hyalommadromedarii* (37.5%) and *Hyalommaexcavatum* (31.3%) are the most prevalent species among the areas.

Table IV. Percentages of the species

	Species	Frequency	Percent	Valid percent	Cumulative percent
Valid	*H.excavatum*	5	31.3	33.3	33.3
	H.dromedarii	6	37.5	40.0	73.3
	H.appendiculatus	1	6.3	6.7	80.0
	R.decoloratus	2	12.5	13.3	93.3
	R.microplus	1	6.3	6.7	100.0
	Total	15	93.8	100.0	
Missing	System	1	6.3		
Total		16	100.0		

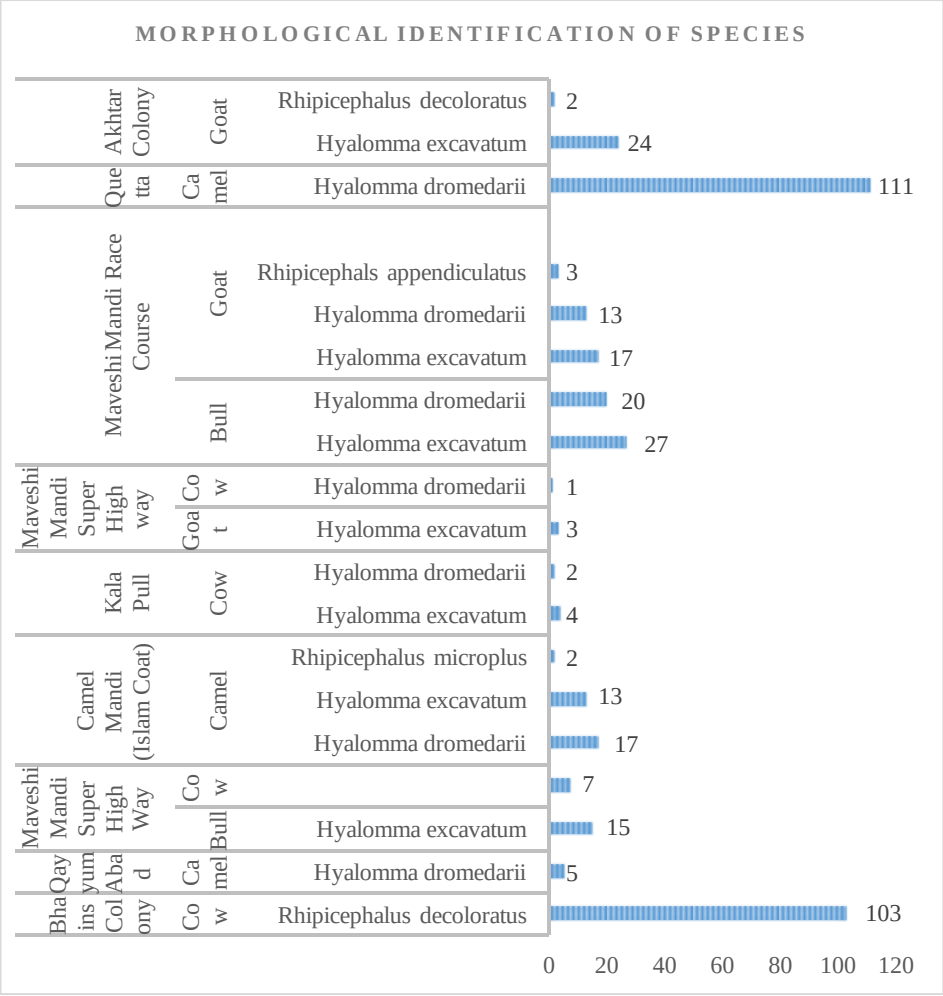

Fig. 4. Morphological identification of species

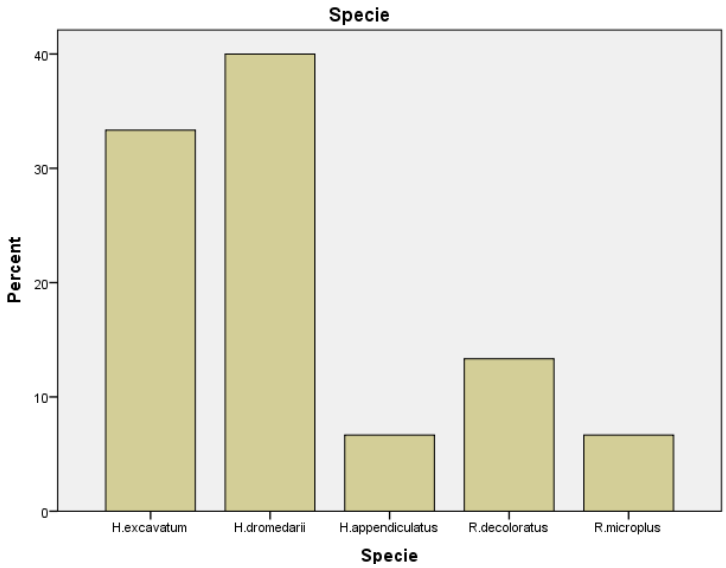

Fig. 5. Graphical Representation showing percentages of prevalent species among areas

PREVALENT SPECIES IN AN AREA

A dataset was extracted for this analysis, and chart was generated (Fig. 6) to enhance graphical representation, clarity, and interpretation (Table V). The analysis revealed that *Rhipicephalus decoloratus* and *Hyalomma dromedarii* were the most prevalent species in Bhains Colony and Quetta, respectively.

Table V. Extracted data from the given data to determine the prevalent specie in an area

Area	Specie	Prevalence
Bhains Colony	*R. decoloratus*	103
Qayyum Abad	*H. dromedarii*	5
MaveshiMandi Super High Way	*H. excavatum*	25
MaveshiMandi Super High Way	*H. dromedarii*	1
Camel Mandi (Islam Coat)	*H. dromedarii*	17
Camel Mandi (Islam Coat)	*H. excavatum*	13
Camel Mandi (Islam Coat)	*R.microplus*	2
Kala Pull	*H. excavatum*	4
Kala Pull	*H. dromedarii*	2
MaveshiMandi Race Course	*H. excavatum*	44
MaveshiMandi Race Course	*H. dromedarii*	33
MaveshiMandi Race Course	*H. appendiculatus*	3
Quetta	*H. dromedarii*	111
Akhtar Colony	*H. excavatum*	24
Akhtar Colony	*R. decoloratus*	2

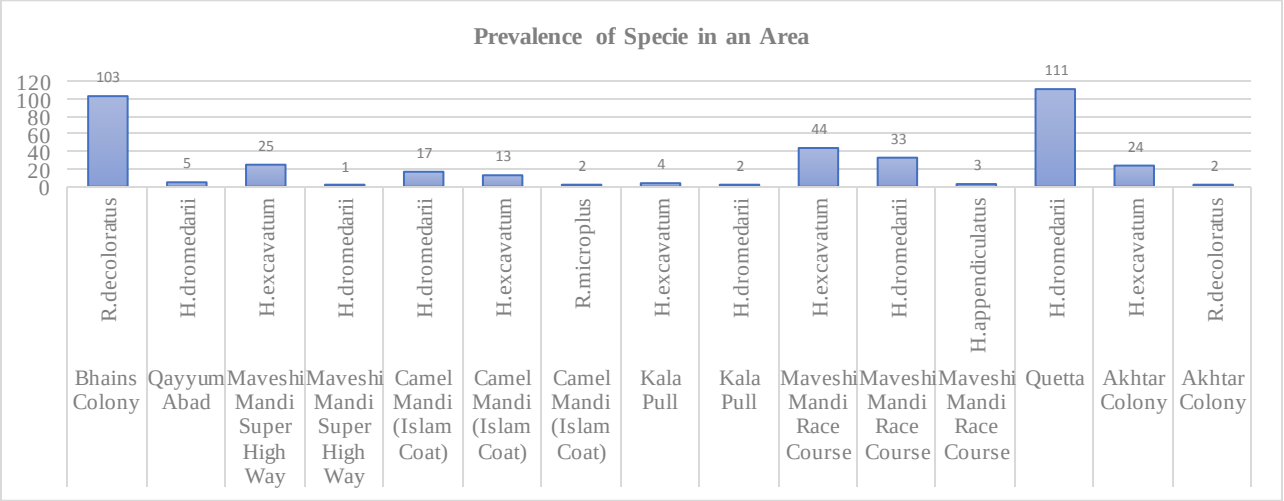

Fig. 6. Graphical Representation Representing Prevalence of Species in an Area

PREVALENT SPECIE IN THE HOST

A Chi-square test was applied to determine the prevalence percentages of tick species across different hosts and results are shown in Table VI. In cattle, *Hyalomma excavatum* and *Hyalomma dromedarii* each accounted for 33% of the identified species. In camels, *H. excavatum*, *H. dromedarii*, and *Rhipicephalus microplus* were present at equal proportions of 33% each. In bulls, *H. excavatum* and *H. dromedarii* demonstrated equal prevalence, each representing 50% of the observed species. In goats, *H. excavatum* constituted 20%, *H. dromedarii* 40%, while *H. appendiculatus* and *Rhipicephalus decoloratus* each contributed 20% of the total tick species identified.

Table VI. Cross tabulation percentages of the species in a Host

			Species					Total
			H. excavatum	*H. dromedarii*	*H. appendiculatus*	*R. decoloratus*	*R. microplus*	
Host	Cow	Count	1	1	0	1	0	3
		% within Host	33.3%	33.3%	0.0%	33.3%	0.0%	100.0%
	Camel	Count	1	1	0	0	1	3
		% within Host	33.3%	33.3%	0.0%	0.0%	33.3%	100.0%
	Bull	Count	1	1	0	0	0	2
		% within Host	50.0%	50.0%	0.0%	0.0%	0.0%	100.0%
	Goat	Count	1	2	1	1	0	5
		% within Host	20.0%	40.0%	20.0%	20.0%	0.0%	100.0%
Total		Count	4	5	1	2	1	13
		% within Host	30.8%	38.5%	7.7%	15.4%	7.7%	100.0%

Excel was also used for visual interpretation to determine the prevalence of species in different hosts as shown in Fig. 7. A dataset was extracted (Table VII) for this specific analysis, and charts were generated to provide graphical representation, enhance clarity, and facilitate interpretation. The results revealed that *Rhipicephalus decoloratus* was the most prevalent species in cows, while *Hyalomma dromedarii* predominated in camels. In contrast, *Hyalomma excavatum* emerged as the most prevalent species in both bulls and goats.

Table VII. Extracted data from the given data to determine the prevalent specie in the host

Host	Specie	Prevalence
Cow	*H. excavatum*	11
Cow	*H. dromedarii*	3
Cow	*R. decoloratus*	103
Camel	*H.excavatum*	13
Camel	*H. dromedarii*	133
Camel	*R. microplus*	2
Bull	*H. excavatum*	42
Bull	*H. dromedarii*	20
Goat	*H. dromedarii*	2
Goat	*H. excavatum*	44
Goat	*H. dromedarii*	13
Goat	*H. appendiculatus*	3
Goat	*R. decoloratus*	2

DISCUSSION

Agriculture is the backbone of Pakistan's economy, with livestock being the most prominent subsector, contributing more than half of the value added by agriculture. According to a 2013 report, Pakistan harbors approximately 170 million domesticated ruminants, often living in close proximity to human populations. A recent study documented 19 tick species infesting livestock across various ecological

regions of Pakistan (3). Among these, three major hard tick genera *Rhipicephalus*, *Haemaphysalis*, and *Hyalomma* were reported to be prevalent (2). In the present study, we identified five species of hard ticks, including two belonging to *Hyalomma* and three to *Rhipicephalus*. These species are recognized as competent vectors of the Crimean-Congo hemorrhagic fever (CCHF) virus, which remains endemic in Balochistan Province. Sporadic spillovers into other provinces are largely attributed to the movement of tick-infested animals from Balochistan (2). Although a six-year study refuted the direct association between CCHF incidence and the Eid-ul-Adha season (6), the casual interaction of human populations with sacrificial animals in Karachi during this period continues to pose a substantial risk of disease transmission.

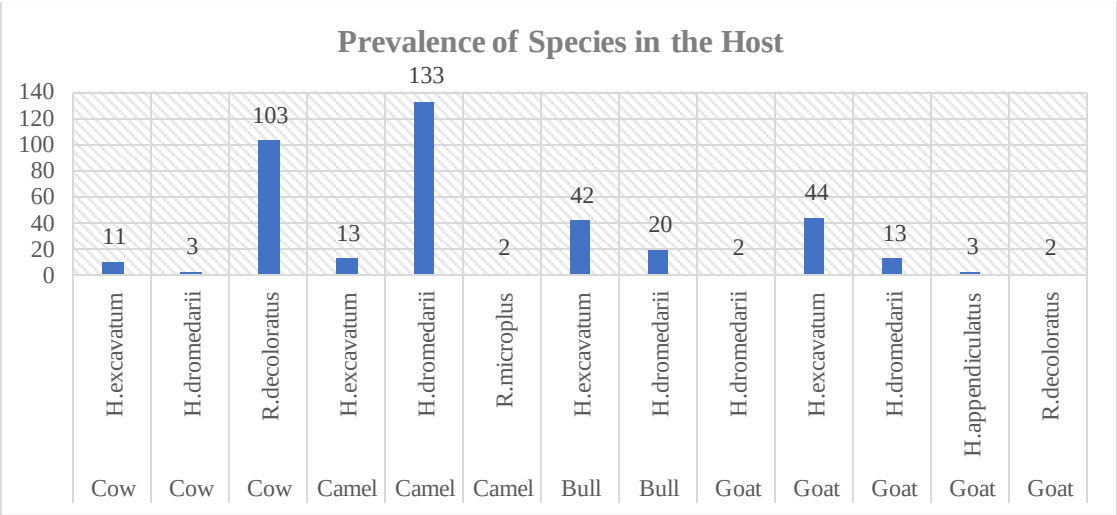

Fig. 7. Prevalence of specie in the host

Our findings are broadly consistent with previous surveys of tick fauna in Pakistan and neighboring countries, which report *Hyalomma* and *Rhipicephalus* as the dominant genera on livestock. Several regional studies from Punjab, Sindh and Khyber Pakhtunkhwa have documented high proportions of *Hyalomma* and *Rhipicephalus* species infesting cattle, buffaloes, sheep, and goats, reflecting similar ecological and husbandry factors that favor these genera. Likewise, multi-country assessments in South Asia and Iran report comparable species assemblages and regionally variable prevalence patterns, supporting the generality of our observations (18-20).

The incorporation of molecular identification in tick surveillance confers several advantages over morphology alone and is particularly relevant to our study. Molecular markers (e.g., cox1, 16S rDNA) resolve cryptic species complexes, provide reliable identification for immature life stages, and enable confirmation where morphological characters are damaged or ambiguous. Additionally, molecular typing facilitates the linkage of tick populations to pathogen detection (e.g., simultaneous screening of tick pools for CCHFV RNA), allows genetic comparison across geographic regions, and supports phylogeographic inference that can reveal routes of tick and pathogen spread. Such molecular approaches have recently uncovered previously unreported *Hyalomma* species and clarified species boundaries in Pakistan and neighboring countries, underscoring their diagnostic and epidemiological value (21).

We propose that this risk can be substantiated through systematic sampling of hard ticks, particularly *Hyalomma* species, from sacrificial animals transported to Karachi from Balochistan and other regions, followed by molecular screening for viral RNA. Our findings demonstrate that hard tick species competent for CCHF transmission are abundantly prevalent in the sampled areas. This situation represents a potential emerging threat that may lead to increased outbreaks in the future, exacerbated by factors such as climate change and rapid population growth. Therefore, adopting integrated control strategies and exploring innovative approaches to tick management in livestock are urgently warranted to address these emerging public health challenges.

CONCLUSION

This study demonstrated that *Hyalomma dromedarii* and *Hyalomma excavatum* were the most prevalent tick species infesting livestock in the sampled regions of Pakistan, while *Rhipicephalus decoloratus*

and other species were detected at lower frequencies. The predominance of *Hyalomma* species, which are recognized as the principal vectors of Crimean–Congo Hemorrhagic Fever (CCHF), underscores their critical epidemiological significance. The findings highlight the potential risk of CCHF outbreaks, particularly during periods of increased human and animal interaction such as Eid-ul-Adha, and emphasize the need for continuous surveillance of tick populations. To minimize this risk, continuous surveillance of tick populations should be prioritized, integrating both morphological and molecular approaches for accurate species identification, including cryptic and immature stages.

Conflict of interest:

Authors declared no conflict of interest.

Authors' contribution:

SU Conceived and supervised the study; RF Performed morphological identification; MZY Conducted molecular analysis. All authors approved the final manuscript.

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