Prevalence, Morphological & Molecular Identification of CCHF Causing Tick species

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Abstract- MThis work 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. Aims: The objective of the work to identify the prevalent of the Tick species sampled from different livestock animals in Pakistan. The Ticks are obligate haematophagous arthropods are obligate commonly distributes throughout the world. The Ticks are the vectors in deadly diseases like Crimean Congo Hemorrhagic Fever (CCHF)). Methods: The study was done in department of Biosciences Shaheed Zulfikar Ali Bhutto University of Science and Technology, Karachi, Pakistan. 389 tick specimens were sampled. The longitude and Latitude of each location recorded by using a hand –held Global Positiontioning System (GPSMAP) Garmin 62S. All samples were observed and morphological identified in the Lab with the help of keys under Stereo and Electron Microscope. Results: Out of total 389 sampled ticks 110 ticks were identified Hyalomma aexcavatum 169, Hylommadromedarii,105, Rhipicephalus decoloratus 2 Rhipicephalus microplus and 3 Rhipicephalus appendiculatus. The percentage of sampled species ticks were H.appendiculatus 0.77%,R. decoloratus 27%,R.microplus 0.5% Hylomma dromadarii 43% and Hylomma excavatum 28% Conclusions: Hylomma dromadarii and Hylomma excavatum found to be the most prevalent tick species infesting the livestock of the sampled sites. High prevalence of these two Ticks species are among the competetent vectors for spreading the CCHF combined with reported cases of the disease in cities of Pakistan.

Index Terms- Ticks, Crimean Congo Haemorrhagic Fever, Livestock.

I. INTRODUCTION

Ticks are obligate blood-suckingecto-parasites belonging to Phylum Arthropoda. Different species of ticks are widely distributed throughout nature and implicated in transmitting a variety of deadly bacteria, protozoal, and viral diseases. Ticks are the vectors in life-threatening [2]. Encephalitis, Mediterranean fever, Crimean Cong Hemorrhagic Fever (CCHF) Siberian Tick Typhaus [1].

Besides transmitting various infectious diseases, ticks also detrimentally affect livestock through infestation leading to reduced productivity and significant economic losses. Ticks have modified their feeding habitat to almost every terrestrial mammal, bird, and reptile [2]. A total of 900 ticks species have been identified so far from which 700 species belong to the Ixodidae (hard/shield ticks) and 200 species belong to the Argasidae (soft/tampans). Ticks are at the number of 2nd only to the mosquito in term of spreading dangerous infectious diseases.[3]

Large-scale analyses of morphological characters and molecular markers of different tick species could reveal important information on their phylogeny.[7]

The objective of the work to identify the prevalent of the Tick species sampled from different livestock animals in Pakistan. The Ticks are obligate haematophagous arthropods are obligate commonly distributes throughout the world. They spread number of Viral, bacterial diseases and are second only to mosquito interims of spreading life menacing infectious diseases. The Ticks are the vectors in deadly diseases like Crimean Congo Hemorrhagic Fever (CCHF)).

inception of ideas till their publications. Research papers are highly recognized in scholar fraternity and form a core part of PhD curriculum. Research scholars publish their research work in leading journals to complete their grades. In addition, the published research work also provides a big weight-age to get admissions in reputed varsity. Now, here we enlist the proven steps to publish the research paper in a journal.

II. METHOS

Field Sampling

Ethical Consent: Verbal consent from the owners of all farm animals was taken prior to sampling.

Sample Collection: Samples were collected between January 2018 and January 2019. Sampling was carefully done using sterile forceps by taking all possible protective measures under field conditions, especially wearing face masks and gloves while dealing with engorged male and female ticks (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].

Temperature/Humidity Range: All environmental parameters related to ticks' habitat like temperature and humidity were recorded in controlled data form. Temperature and relative humidity range during the sampling were found to be 32°C – 40°C and 32% – 57% respectively.

GIS Mapping: The latitude and longitude of sampling locations in Karachi city were recorded using a hand-held Global Positioning System (GPSMAP) Garmin 62S.

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 by11 Stereo and Electron Microscope: Ticks identification was carried out under the stereo and electron microscope (figure 1-2).

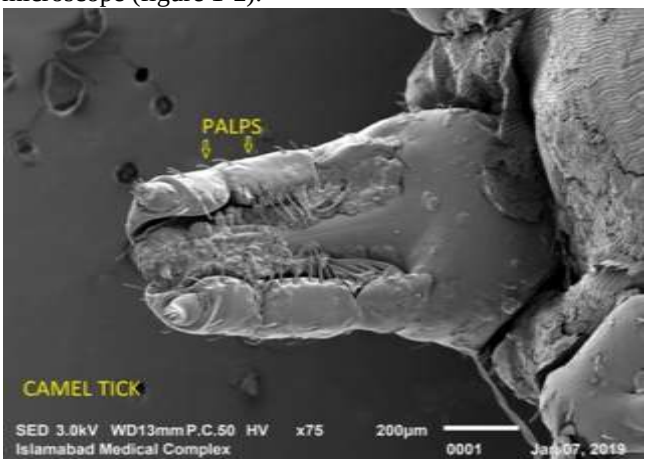

Figure 1: Electron microscopic image of tick mouthpart

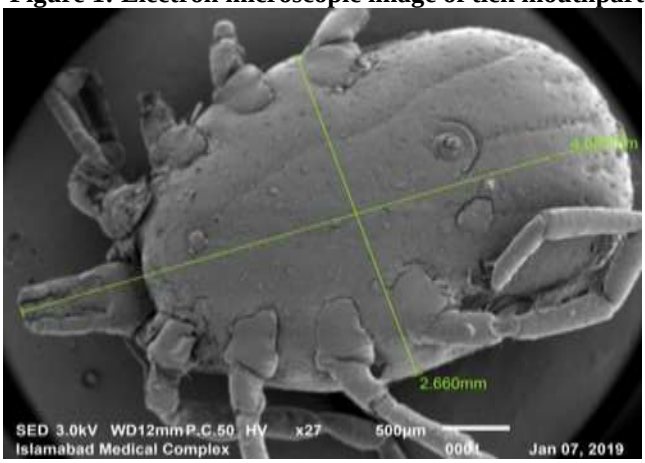

Figure: 02 Electron microscopic image of tick Diaphragm

Pooling of Samples: After morphological identification, most of the samples were pooled (based on species, gender, and location) and frozen at -80°C for CCHF virus screening by RT PCR. Few representative individual specimens were preserved for species confirmation by molecular methods.

3.7 Molecular Identification of Tick Species

3.7.1 Homogenization

All steps given below prior to inactivation (i.e. before adding lysis buffer to the tick sample homogenate) are to be performed in a Biosafety Cabinet Class II with in ABSL-3 containment or prescribed otherwise for specific virus [8].

The tick samples were transferred to a 2 mL OMNI bead rupture tube. The original sample tube rinsed with concentrated household bleach (Sodium Hypochlorite, Available Chlorine ~5.0%) prior to disposing of it in the solid waste receptacle [9].

All micropipette tip waste generated before the inactivation steps was decontaminated with concentrated bleach by pipetting it up and down several times prior to disposing of it in the solid waste receptacle. The tick sample was washed twice with 1 mL sterile PBS pH 7.4 and discarded in a liquid waste receptacle containing concentrated household bleach (~5.0%).

Eight stainless steel beads of 2.4 mm (pre-sterilized at 250°C for 2 hours) were added to the tube containing the washed tick sample, and then 1 mL sterile PBS pH 7.4 added to a tube containing the tick sample and beads.

Preferably cool down the temperature of OMNI Bead Ruptor (Homogenizer) using Liquid Nitrogen or Ethanol Dry Ice Mixture in the connected BR Cryo Unit.

The temperature of the homogenizer cooled down using ice in the BR Cryo Unit assembly.

Before loading the sample tube into the homogenizer, ensure that the tubes are appropriately capped.

Used carriage for 2 mL tube, run 2 cycles of the following program:

- S = 6.
- C = 10
- T = 0:10
- D = 0:15

the tube cooled down on ice for 5 minutes after the end of each cycle. The Centrifugation at 4000-5000 rpm was done for 10-12 minutes to clarify the homogenized sample, then 0.25 mL of supernatant transferred to a new 1.5 mL nuclease-free micro centrifuge tube for DNA extraction.

Extraction of DNA: Extracted DNA from Tick by using a commercial DNA extraction kit (Gene Jet Genomic DNA extraction Kit, K0721) as per manufacturer protocol.

Amplification of the second internal transcribed spacer gene (ITS2) by PCR

Gently vortexed and briefly centrifuged Hot Start Green PCR Master Mix (2X) after thawing.

Added the following components for each 25 µl reaction at room temperature:

- Hot Start PCR Master Mix (2X) 12.5 µL
- ITS-F (10 pmol/µL) 1 µL
- ITS-R (10 pmol/µL) 1 µL
- Template DNA 2 µL
- Nuclease Free Water 8.5 µL
- Total volume 25 µL

Table 1: Primer Sequence

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

Prepared Template Control 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).

Gently vortexed the samples and spun them down Performed PCR using the recommended thermal cycling conditions outlined below:

Table 2: Cycling Conditions

Step	Temperature C	Time	Number of cycles
Initiation denaturation	95	4 min	1
Denaturation	95	30s	**40**
Annealing	57	30s	
Extension	72	50s	
Final Extension	72	5-7min	**1**

Agarose Gel Electrophoresis

Directly loaded 5–10 µL with loading dye of the amplified product on to 1.5% agarose prepared in 1 x TBE or 1 x TAE buffer containing 0.5 µg/mL ethidium bromide. Used appropriate 100bp DNA ladder as reference DNA size marker. Performed electrophoresis in 1X TBE or 1X TAE buffer for 60 minutes at 5V/cm. Visualized amplified product and determined the size of amplified product using gel documentation system (figure 3).

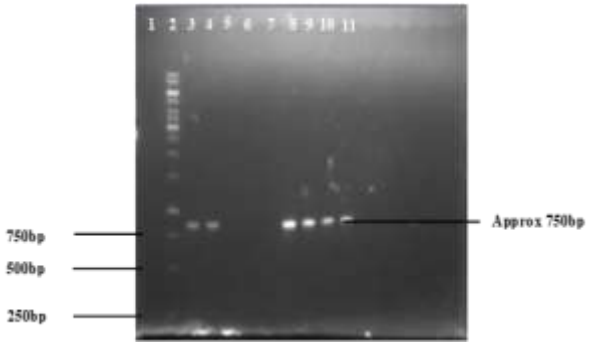

Figure 3: Tick (750bp) PCR Product (Internal Transcribed Spacer (ITS-2) Genes)
Lane 1:1kb DNA ladder (Thermoscientific, SM0313)
Lane 2, 3, 7 to 10: PCR Product Tick (750bp)
Lane 11: Negative Control

Purification of amplified DNA product

Purify desired amplified DNA product from Agarose gel using Thermo Scientific Gene jet Gel Extraction Kit #K0691, as per manufacturer protocol.

III. 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). The percentages of the species show that Hyalommadromedarii (37.5%) and Hyalommaexcavatum (31.3%) are the most prevalent species among the areas. Rhipicephalusdecoloratus and Hyalommadromedarii are the most prevalent species in areas like Bhains Colony and Quetta, respectively (Table1).

Hyalomma dromedariiand Hyalomma excavatumare the most prevalent species present in all hosts including, Cow, Camel, Bull, and Goat, and are found in all areas except Bhains Colony. Hyalomma decoloratusis significantly prevalent in host like Cow in an area of Bhains Colony while it is non-prevalent in Goat found in Akhtar colony area. Rhipicephalus microplusis non-prevalent and can be found in Camel in an area like Camel Mandi (Islam coat). Rhipicephals appendiculatusis a non-prevalent specie found in Goat in an area like Maveshimandi race course. Hyalomma dromadarri, 43%, and Hyalomma Excavatum, 28% were found to be the most prevalent Ticks species infesting the live stock of the samples sites (Table: 2).

Table: 1 Total number 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	_	_	_	_	_
MaveshiMandi Race Course	22	_	_	_	_
MaveshiMandi Super Highway	13	17	_	_	2
Kala Pull	4	2	_	_	_
Camel Mandi Islam Coat	3	1	_	_	_
MaveshiMandi Super Highway	44	33	3	_	_
Qayyumabad	_	111	_	_	_
Bhains Colony	24	_	_	2	_
Total	**110**	**169**	**3**	**105**	**2**
Percentage	**28%**	**43%**	**0.77%**	**27%**	**0.5%**

Table: 2 Percentage (%) of Tick species in different counties of Quetta and Karachi

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

In Cow the percentages of

species like *Hyalomma excavatum* and *Hyalomma dromedarii* are 33%. In Camel the percentages of species like *Hyalomma excavatum*, *Hyalomma dromedarii* and *Rhipicephalus microplus* are 33%. In Bull the percentages of species like *Hyalomma excavatum* and *Hyalomma dromrdarii* are 50%. In Goat the percentage of *Hyalimma excavatum*is 20%, *Hyalomma domedarii*is 40%, *Hyalomma appendiculatus* and *Rhipicephalus decoloratus* are 20%.

The most prevalent specie in Cow is Rhipicephalus decoloratus. The most prevalent specie in Camel is Hyalomma domedarii. The most prevalent specie in Bull and Goat is Hyalomma excavatum (Figure 4).

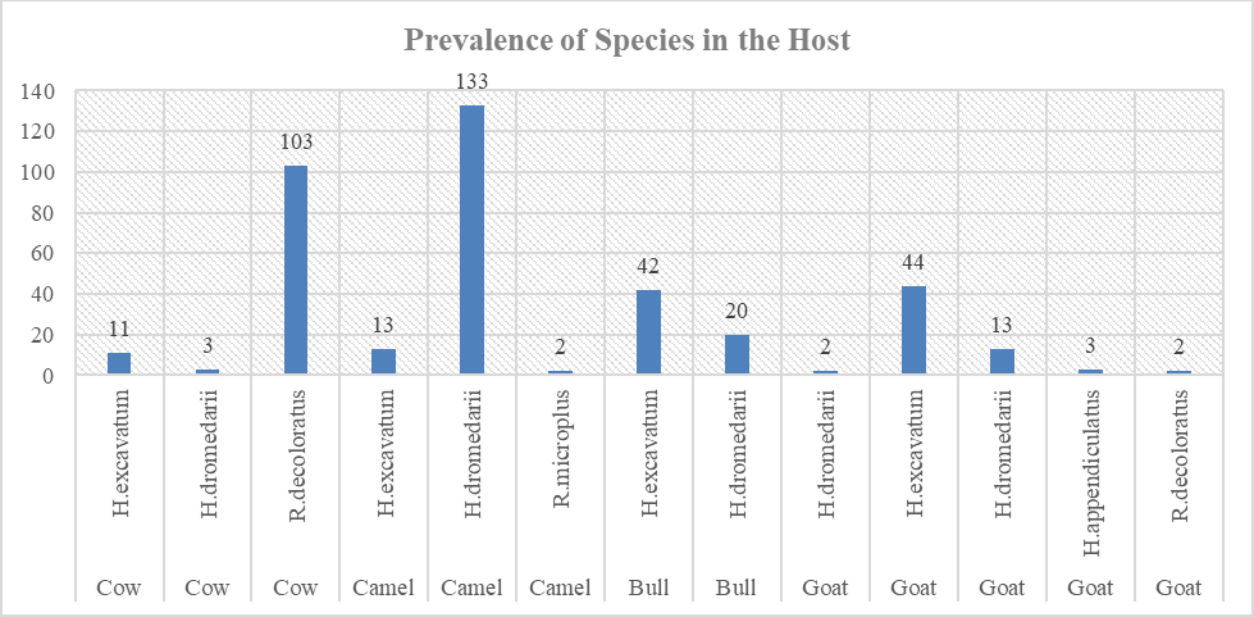

Figure 4 *Prevalence of Specie in the Host*

IV. DISCUSSION

Agriculture is the backbone of Pakistan's economy and Livestock among other subsectors is undeniably the most prominent one as it shares more than one-half of the value added by agriculture. According to a 2013 report, Pakistan is home to around 170 million domesticated ruminants living in close proximity to the human population. A recent study reported 19 tick species infesting livestock in different ecological regions of Pakistan [3]. Three important hard ticks i.e. Rhipicephalus, Haemaphysalis, and Hyalomma are reported to be prevalent in Pakistan [2] In the present study, we came across five species of hard ticks, of which two belonged to Hyalomma and three belonged to Rhipicephalus. These species are notorious for being competent vectors of the CCHF virus as evidenced by an endemic state of the disease in the Balochistan Province of Pakistan. Occasional spillovers of the disease in other provinces of Pakistan are attributed to the movement of tick-infested animals from Balochistan [2].

Although the notion of a direct association between the occurrence of CCHF cases and the time of Eid-ul-Adha based on a 6-year study was rebutted [6].

We still consider that casual interaction of human populations with the sacrificial animals in Karachi city during Eid-ul-Adha season will always pose a threat of way more than a few isolated disease incidents. We propose to corroborate this by sampling hard ticks, especially Hyalomma species, from sacrificial animals brought to Karachi from Balochistan and other parts of the country and screening them for selected viral RNA portion.

V. CONCLUSION

The present study showed that hard tick species competent to transmit CCHF are abundantly prevalent in the sampled region. This in our view corresponds to a time bomb that may culminate in increased disease incidents in the future due to worsening factors like drastic climatic changes and ever ever-increasing human population. Adopting integrated approaches and researching innovative ways of tick control in livestock are clearly warranted to meet the emerging challenges.

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