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PREVALENCE OF *THEILERIA ANNULATA* INFECTED HARD TICKS OF CATTLE AND BUFFALO IN PUNJAB, PAKISTAN

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ABSTRACT

A study to investigate prevalence of theilleria infected hard ticks of dairy cattle and buffalo was carried out in Faisalabad, Jhang and Khanewal districts of Punjab, Pakistan. Ticks were collected during July and August, 2007 from infested dairy animals irrespetive of age, sex and weight. These animals were from 30 livestock farms, each having more than 25 animals (cattle and buffaloes). Six thousand two hundred and sixty three ticks were collected from 710 cattle and 320 buffaloes. Overall prevalence of *Hyalomma* species was significantly higher (61%) than that of other genera of hard ticks ($p>0.05$). In sex-wise distribution, it was found that female *Hyalomma* species were the highest (85%) followed by *Amblyomma* species (81%), while prevalence of *Boophilus* species and *Haemaphysalis* species were 77%. The ticks infestation rate in cattle (70%) was significantly higher than that of buffaloes (34%). PCR results showed that *Theilleria annulata* was detected in 50% *Hyalomma anatolicum* and 40% *Hyalomma dromedary* ticks. No *theilerial* organism was detected in *Hyalomma marginatum*, *Boophilus annulatus* and *Amblyomma varigatum* ticks.

In conclusion, Theilleria annulata was prevailing in more than 40% Hyalomma species of cattle. It means that Hyalomma species are mainly responsible for transmission of the Theileria in dairy animals.

Key words: Prevalence, Buffaloes, Hard ticks, Livestock census.

INTRODUCTION

Ticks are obligatory blood-sucking ectoparasites infecting mammals, birds, reptiles and amphibians. There are three families of ticks but Ixodidae (hard ticks) and Argasidae (soft ticks) are of veterinary importance (Sonenshine, 1991). Eighty percent of the world tick fauna are hard ticks and with the exception of one tick species in family Nuttalliellidae, the remaining are soft ticks. Ten percent of the total hard and soft tick species are known to cause disease transmission in domestic animals and humans (Jongejan and Uilenberg, 2004). Tick species that remain on the host during two molting periods are known as one-host ticks. In two host species, the larvae molt to the nymphal stages occur on the host but engorged nymphs leave the host, molt in the environment and then find a new host. In the three host tick life cycle, both the larvae and nymph leave the host to molt, attaching to host again after each molt (Zajac *et al.,* 2006)

It has been studied that about 80% of the world cattle population is infested with ticks (Bowman *et al.,* 1996). Food and Agricultural Organization of the United Nations estimated the global losses of hard tick infestation to be US $ 7.0 billion annually (Harrow *et al.,* 1991). Moreover, ticks can cause transmission of viral, bacterial and protozoan pathogens causing diseases like hemorrhagic fever, ehrlichiosis, anaplasmosis, theileriosis, and babesiosis in meat and dairy animals (Rajput *et al.,* 2006, Table-1). The ticks suck host blood during their lengthy attachment period (7-14 days), which may be extended depending on the tick species and unique host association (El Hakim *et al.,* 2007). Tick infestation diminishes quality of skin/hide up to 20-30%. (Gharbi *et al.,* 2006) and causes severe anemia, loss of production, weakness and immunosuppression in the infected animals (Gwakisa *et al.,* 2001).

Optimal relative humidity and temperature requirements for growth and reproduction of ticks are 85% and 26-37^{o}C, respectively (Aktas *et al*., 2004). Yakhachali and Hosseine (2006) described the seasons of highest tick prevalence as fall and winter at 50%, the least being spring and summer. Meteorological data of the study area can be correlated with the months of study in the area.

Various methods which include application of acaricides, injectables, oral medication (herbal), burning of pastures and selection of tick resistant breeds are being used to control ticks. Application of acaricides is the most common way to minimize the problem and is applied in the form of spray, shower/spot on and dip. Due to widespread use of these chemicals, acaricide resistant ticks are being emerged (Nolan, 1990).

Data regarding prevalence of theileria infected hard ticks is important to devise necessary control program for ticks and theileria infection. This study was therefore conducted to collect ticks from cattle and buffaloes in three districts (Faisalabad, Jhang and Khanewal) situated closely in central Punjab, Pakistan. The ticks were characterized up to species level and also processed for PCR based detection of theileria species.

MATERIALS AND METHODS

Study Area: This study was conducted at 30 randomly selected livestock farms of districts Faisalabad, Jhang and Khanewal in the Punjab province, Pakistan. The area is canal irrigated and heavily populated with livestock. Total livestock population in these three districts is estimated to be 1.75 million cattle (local, cross bred and exotic breeds), 2.88 million buffaloes, 0.59 million sheep and 1.98 million goats (Ahmad *et al.,* 2000). Cattle on these farms are mostly cross bred and exotic and kept in cemented and bricked farm buildings.

Collection of Ticks and tick infestation level: The ticks were collected in morning and evening in the months of July and August, 2007 from 710 cattle and 320 buffaloes. Tick infestation rate and tick infestation level were also estimated in all these bovine, 10 farms from each of three districts were selected randomly for these studies. Tick infestation level study was performed by categorizing cattle and buffalo population into three levels i.e. low, moderate and high infestation levels. Animals having 1-25 ticks were designated as low infestation level, while animals having 26-50 and above 50 ticks were characterized as moderate and high infestation levels, respectively. With the help of small forceps ticks were collected systemically as per Muhammad *et al.* (2008) starting from head towards tail direction and placed in a Petri dish. Care was taken to avoid decapitulation and shedding of legs. The tick samples were dispatched to Parasitology laboratory in clean and dry properly labeled plastic bottles. The mouths of these plastic bottles were covered by cheese cloth for proper aeration.

Tick collection for the *T. annulata* detection: Twenty morphologically speciated ticks were taken from each of five species (*Hyalomma anatolicum, H. dromedari, H.marginatum, Boophilus microplus and Amblyomma vareigatum*) and were used for detection of *T. annaula* by PCR. Tick collection for this experiment was made in sterile glass bottles from cross bred cattle.

Identification of Ticks: In the laboratory, the ticks were kept in 70% ethyl alcohol for the purpose of preservation. The collected ticks were characterized microscopically on the basis of morphology with the help of dichotomous key described by Hoogstral (1956).

Separation of female hard ticks from the male: After genera identification of collected ticks, female adult ticks of each genus were separated by observing small area of scutum on the anterior dorsum of each tick (Urquhart *et al.,* 1996).

Primers: Primers (IA-1 and IA-2: First base, Singapor) were used for the amplification of the gene coding for surface protein of the merozoite (Table-2).

Table-2 Oligonuleotide primers used for amplification in PCR Analysis

Primer	Sequence	Position	Expected Amplified DNA length
IA-1 (R)	5'-GTAACCTTTAAAAACGT-3'	142-158	721
IA-2 (F)	5'-GTTACGAACATGGGTTT-3'	862-846	

DNA was extracted from ticks by Phenol-Chloroform method as described by d'Oliveira *et al.,* 1997 and 2ul of extracted DNA was used for PCR reaction. DNA quantity was measured at 260 nm. The ratios of 260/280 were ranging from 1.80 to 1.95 for purified DNA samples which showed that preparations were free of any major protein. After quantification, DNA samples thus extracted were stored at -40^{o}C till used for PCR analysis.

Preparation of final reaction volume and PCR analysis: The mixture for PCR was prepared in volume of 50ul containing 200uM of dNTPS, 1.5mM of $MgCl_2$, 10mM of Tric HCl, 50mM of KCl, 200pM of each of reverse and forward primer, 0.1% of Tritone and 2.5 IU units of Taq DNA polymerase. The temperature cycles used for PCR analysis are: initial heating for five minutes at 94^{0}C, following by 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for one minute. Final extension step was programed at 72^{o}C for seven minutes. Two controls, one negative and one positive were included in each test. The reaction mixtures were performed in automatic conventional DNA thermocylcler (Esco Technologies, Inc.: USA). The amplified samples were electrophoresed on 1.0 % agarose gel (Research Organics, Inc. USA) containing 1X Tris-acetate EDTA buffer containing 0.1ug/ml of Ethidium bromide and were saved after UV light visualization.

RESULTS

Faisalabad, Jhang and Khanewal are three closely located districts in the Punjab province of Pakistan, which is also known as Central Punjab. Central Punjab is canal irrigated area and has fertile land which supports agriculture and livestock farming. We selected Faisalabad, Jhang and Khanewal as these districts are considered as top livestock population districts in Pakistan. In the region people live in rural areas and depend upon crop agriculture and livestock for their livelihood. These districts have similar weather conditions around the year (Fig 1, Fig 2 and Fig 3) as they are located closely in hot and humid Central Punjab

(Fig-4). Tick infestation prevalence in these areas is high due to favorable humidity and temperature conditions, particularly during hotter months of the year.

This study was conducted in the months of July and August, 2007. Temperature and humidity during study period were 30-35^{o}C and 60-70%, respectively. In all three districts *Hyalomma* species were found the highest in prevalence at 61% as compared to other species (Table-3). Faisalabad district was the highest for *Hyalomma* species being 43% followed by Jhang at 28% and Khanewal at 29%. *Boophilus* species were the second most prevalent species with an average prevalence of 28%, Faisalabad being the highest at 66% followed by Jhang at 26% and Khanewal at 7%. *Amblyomma* species were the third major species detected (average 8%). This tick species was the highest in Jhang at 86% compared to Faisalabad at 8% and Khanewal at 6%. *Haemaphysalis* species were the lowest at 3% in prevalence in three districts; Jhang being the highest at 58% followed by Faisalabad at 18% and Khanewal at 24%. *Rhipecephalis* species were not detected in these districts in our study . In sex-wise distribution of hard ticks, adult female ticks were significantly higher compared to adult male ticks and they were at 85% and 81% for *Hyalomma* species and *Amblyomma* species, respectively. *Boophilus* species and *Haemaphysalis* species have similar female to male ratio as 77% of adult ticks were females.

Tick infestation rate in cattle and buffalo population were also estimated in the districts and compared. It was found that infestation rate was significantly higher in cattle (70%) compared to buffaloes (34%). Tick infestation level study showed that high infestation level (tick number above 50) was in 59% cattle population compared to 18% buffalo population. Moderately infestation level (tick number 25-50) was in 38% buffalo and 23% in cattle. In low level infestation (1-25), it was found in 44% buffaloes and 18% in cattle.

PCR based detection of *Th. annulata* was performed from 100 ticks randomly selected to represent the species of ticks (20 ticks per tick species). The prevalence of *Th.annulata* in *Hyalomma anatolicum* and *H.dromedari* ticks was 50% and 40% respectively. No theilerial organism was detected from *H. marginatum, B. annulatus* and *Amblyomma varigatum* ticks. (Table-4, Fig-5).

Table-1: Some tick transmitted protozoan, bacterial and viral diseases with their hosts (Rajput *et al*. 2006 & Jongejan and Uilenberg, 2004)

	Pathogen	**Disease with host**
Protozoa	*Theleiria annulata*	Bovine theileriosis (cattle)
	Th.parva	East coast fever(cattle)
	Bebasia bovis	Bovine babesiosis (buffalo and cattle)
	B. caballi	Equine babesiosis (horse)
	B. canis	Canine babesiosis (dog)
	Anaplasma marginale	Bovine anaplasmosis (cattle)
Bacteria	*Ehrlichia chaffeensis*	Human ehrlichiosis (man)
	E.ruminatum	Cowdriosis (ruminants)
	Borrelia bugdorferri	Lyme Disease (man)
	Francisella tulerensis	Tuleraemia (animals, man)
Virus	Kyasnur Forest Virus (flavivirus)	Kyasnur Forest Disease (man)
	Cremian Congo Haemmorhagic virus (Bunya virus)	CCHV disease (man)
	Tick borne meningoencephalitis virus (flavi virus)	Tick borne encephalitis (animals, man)

Table-3 Genus-wise distribution of hard tick in three districts of Punjab

Name of ticks	**Prevalence percentage in three districts (%)**		
	Faisalabad	**Jhang**	**Khanewal**
Hyalomma	43	28	29
Boophilus	66	26	7
Amblyomma	8	86	6
Haemaphysalis	18	57	24
Rhipicephalus	0	0	0

Table-4 Prevalence of *Th. annulata* in different hard tick species by PCR Analysis

Name of Species	No. of ticks		Positive ticks		Prevalence %		Overal Prevalence%
	Female	Male	Female	Male	Female	Male	
H.analiticum	10	10	7	3	70	30	50
H.dromedari	10	10	3	1	30	10	40
H.marginatum	10	10	0	0	0	0	0
B.annulatus	10	10	0	0	0	0	0
A.variegatum	10	10	0	0	0	0	0

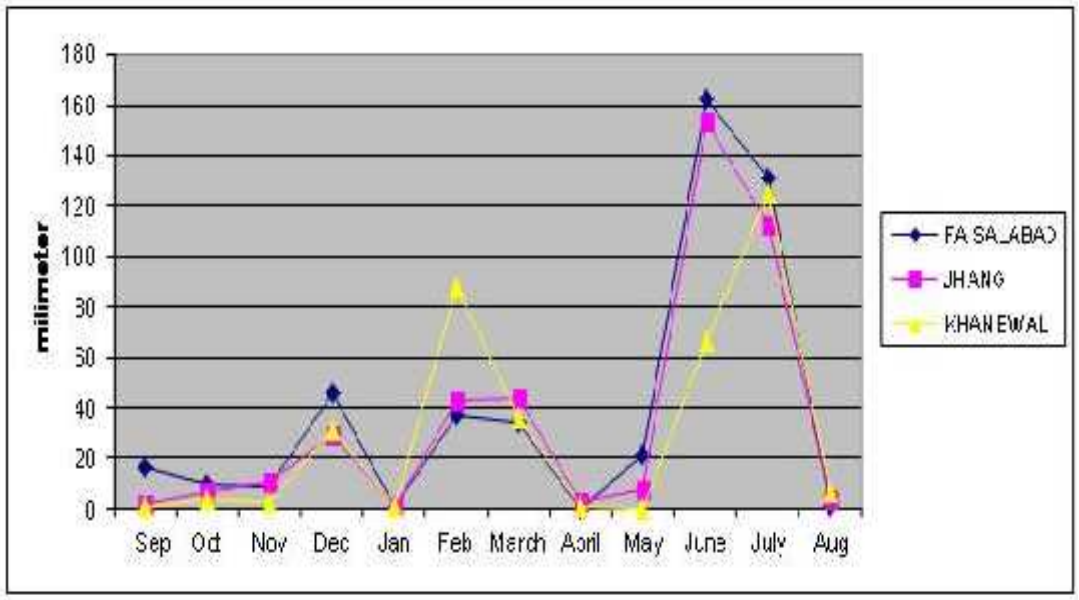

Fig-1 Monthly rain fall in three districts of Punjab

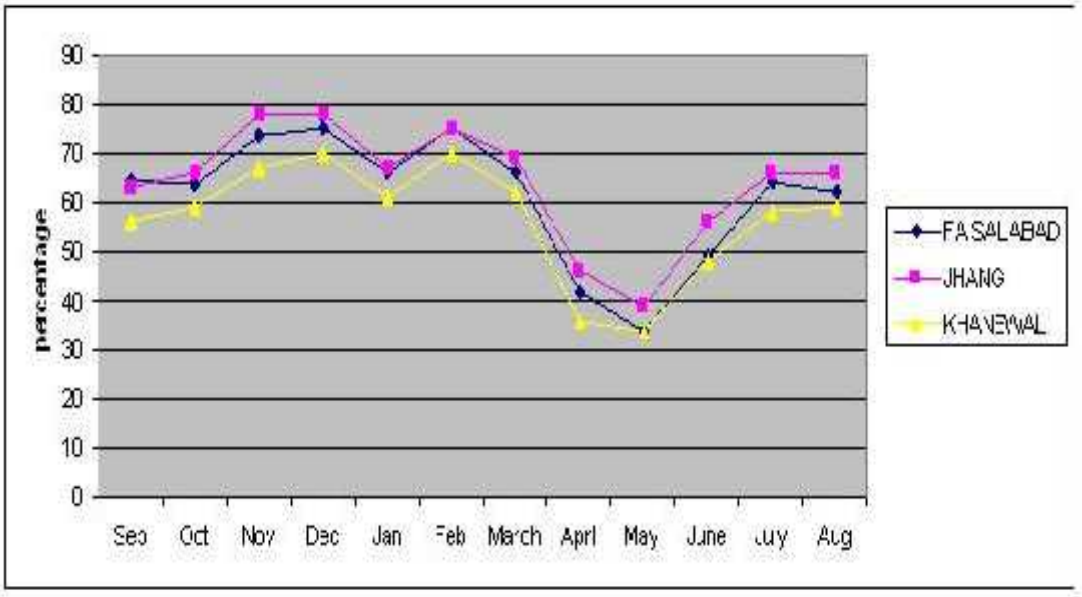

Fig-2 Mean Monthly humidity in three districts of Punjab

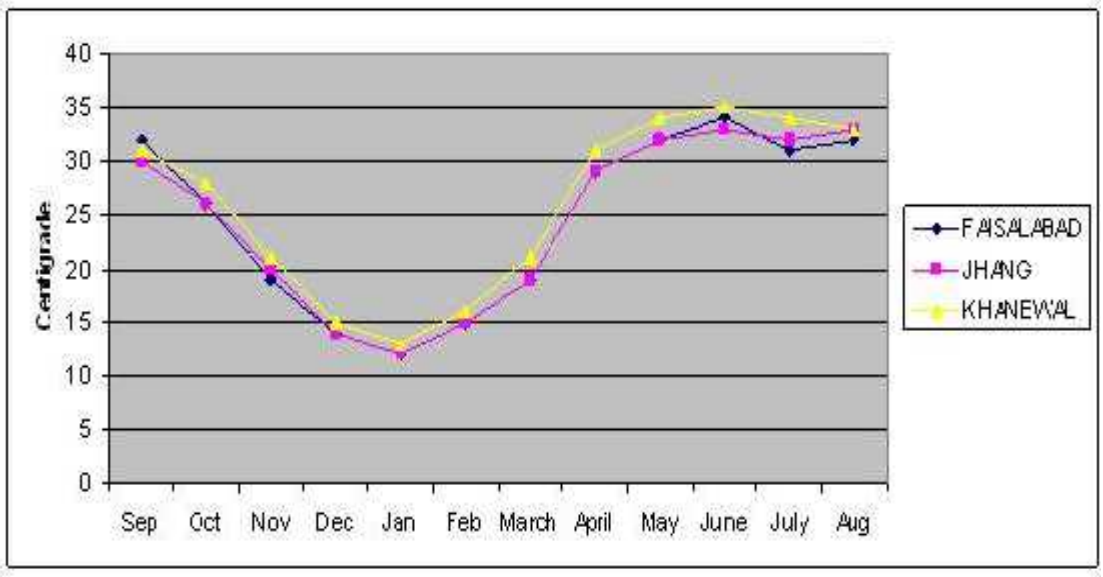

Fig-3 Mean monthly temperature in three districts of Punjab

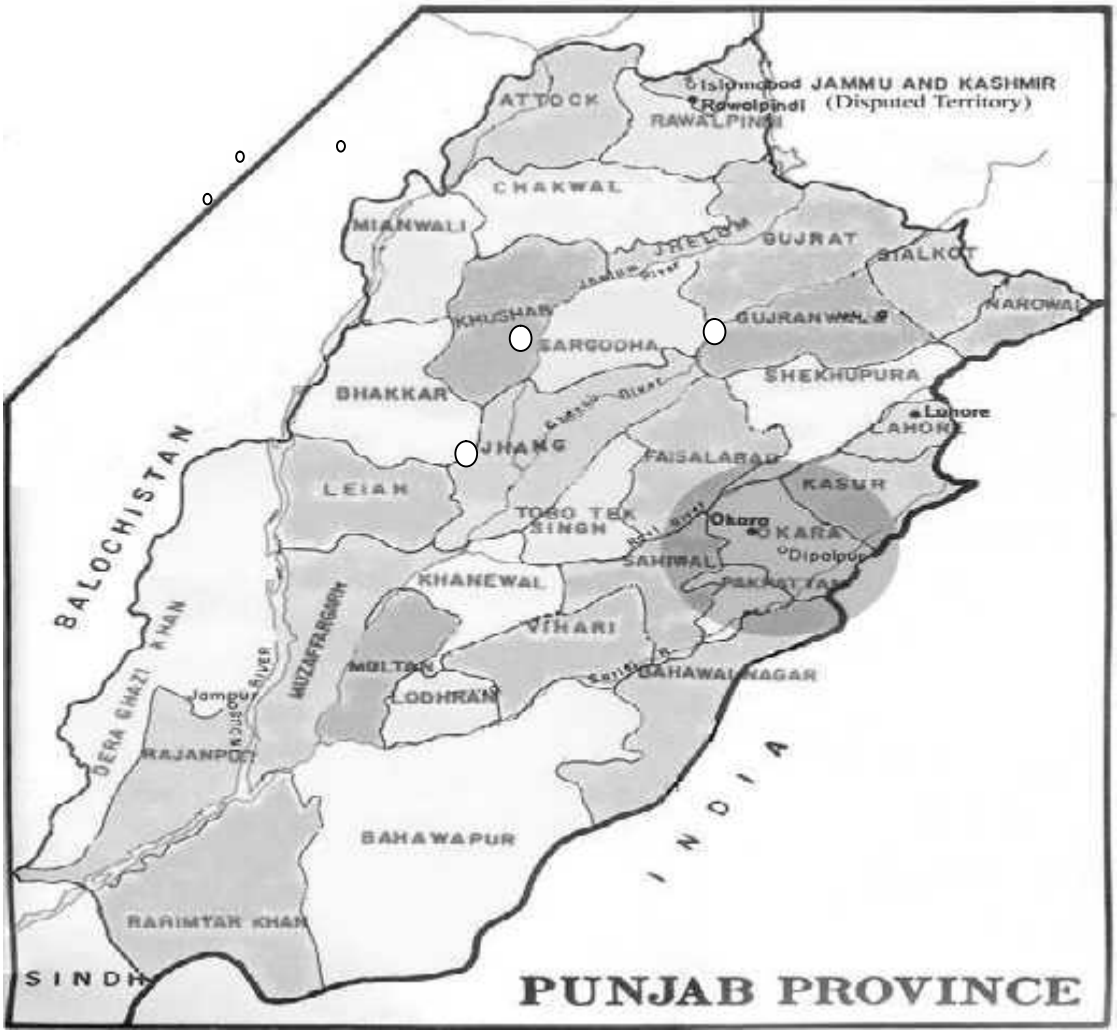

Figure-4. Map showing tick collection districts (Faisalabad, Jhang and Khanewal).

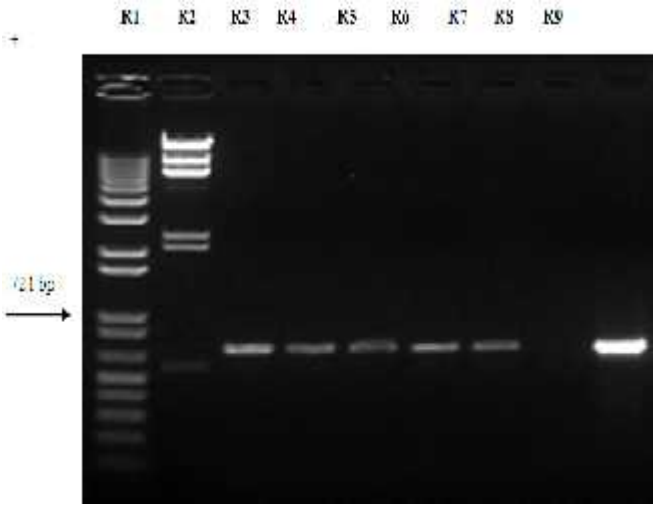

Figure-5. Analysis of amplified product of samples taken from ticks with primers IA-1 and IA-2

R1= 1Kb + DNA ladder, R2= Lambda hind III, R3, R4, R5, R6, R7= positive samples of Th. annulata (N516/N517), R8= negative sample, R9= positive control

DISCUSSION

Tick infestation is a common problem of dairy cattle in Punjab, Pakistan. Prevalence of *Boophilus* species*, Haemaphyslis* species and *Rhipecephalus* species were 8.1%, 5% and 3.1%, respectively. Ticks infestation is a common problem in domestic animals of Rawalpindi, Lahore and Multan districts of Punjab, Pakistan. Prevalence rate of *Hyalomma* species (12%) that was very low previously in Punjab and *Amblyomma* species were not reported (Durrani and Shakoori 2009). However, prevalence of *H. anatolicum* is high (41%) in cattle population in districts of Muzaffargarh and Layyah and prevalence rate of *R. sanguineus* is 25.5%, while prevalence rate of *H. anatolicum* in buffalo population is 36.3%. Prevalence of *Hyalomma* species is higher than that of other tick species (Sajid *et al.* 2008). In Peasawar region in Khyber Pakhtunkhwa province of Pakistan, prevalence of *Boophilus* species, *Hyalomma* species, *Rhipicephalus* species and *Amblyomma* species was 46%, 31.25%, 17.9% and 4.6%, respectively (Manan *et al.* 2007). In Rawalpindi and Islamabad area, *Boophilus* species*, Amblyomma* species and *Rhipicephalus* species are not reported but prevalence of *Haemaphysalis* species is 74% and *Hyalomma* species is 26%. (Rehman *et al.,* 2004).

Present study showed that prevalence of female adult ticks was significantly higher than male ticks. The information is in accordance to Sayin *et al*. (2003) who recorded higher distribution of female ticks compared to male ticks. Prevalence of female tick population of *Hyalomma* species was higher than male ticks (Flach *et al.* 1993 and Aktas *et al.* 2004). In Bangladesh, prevalence of *Boophilus* species and *Haemaphysalis* species is 77% and 72%, respectively (Ghosh *et al.* 2007). Salivary gland of adult female *Hyalomma* ticks have more type III acini compared to male and *Theileria* species are only found in Type III acini. This could be plausible reason that female ticks has more *Theileria* transmission potential as compared to male *Hyalomma* tick (Young *et al.,* 1983). Moreover, histamine binding proteins which counteract pain/response of host to tick attachment are two in female tick compared to male ticks (Bross and Wikel, 2004).

Intensity of tick infestation in cattle was higher than that of buffaloes. In tick infestation level study (tick number above 50), it was significantly higher in cattle (59%) compared to buffalo (18%). Moderately infestation level (tick umber 25-50) was higher in buffaloes (38%) compared to cattle (23%) while in case of low infestation level (1-25), it was significantly higher in buffalo (44%) compared to cattle (18%). Tick infestation is recorded in 75% cattle and 40% buffaloes (Sajid *et al.,* 2008). Double magnitude of ticks in cattle are found compared to buffaloes (Rehman *et al.* 2004 and Manan *et al.* 2007). High tick infestation rate and high tick level infestation in cattle may be due to the reason that majority of cattle in the area of study were cross bred which are more vulnerable to tick infestation (Fesharki, 1988).

Theileria annulata was only detected in *H. annatolicum* and *H. dromedari* but not in *H. marginatum. B.annulatus and A.variegatum.* High incidence of theileriosis in the districts under investigation may be the indication of high infestation of *Hyalomma* species (Khan *et al.,* 2004).

It is concluded that prevalence of *Hyalomma species* of hard ticks in cattle and buffaloes is significantly higher than other species of the ticks. More over, *Theileria annulata* is prevailing in more than 40% *Hyalomma* species of cattle. It indicated that only *Hyalomma* species of ticks are spreading *Theileria* infection amongst cattle population.

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