Aus dem Friedrich-Loeffler-Institut, Greifswald – Insel Riems, und dem Institut für Parasitologie und Tropenveterinärmedizin der Freien Universität Berlin

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Epidemiology of Ticks and Tick-borne Pathogens in the Semi-arid and the Arid Agro-ecological Zones in Pakistan

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

an der

Freien Universität Berlin

vorgelegt von

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Tierarzt aus Multan, Pakistan

Berlin 2016

Journal-Nr.: 3932

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus):
Ticks; tick-borne pathogens; anaplasmoses; ehrlichioses; rickettsial diseases; babesiosis; theileriosis; risk factors; livestock; Pakistan; epidemiology

Tag der Promotion: 14.12.2016

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-781-1
Zugl.: Berlin, Freie Univ., Diss., 2016
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

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Thesis submitted
for the fulfillment of a doctoral degree in Veterinary Medicine at
the Freie Universität Berlin

Submitted by
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This dissertation is dedicated to
my beloved mother, father, brothers and sisters
and to my beloved wife

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Abbreviations

A.	*Anaplasma*
AEZ	Agro-ecological zone
AFS	Australian Friesian-Sahiwal
AI	Aridity Index
AIC	Akaike information criterion
AIPS	American Institute of Pakistan Studies
AK	Azad Kashmir
ALPS	Agro-livestock Production System
Am	*Amblyomma*
AMZ	Australian Milking Zebu
APLPS	Agro-pastoral Livestock Production System
B.	*Babesia*
Bo.	*Boophilus*
bp	Base pair
CCHF	Crimean-Congo haemorrhagic fever
CDC	Centers for Disease Control and Prevention
CGIAR	Consultative Group for International Agricultural Research
CI	Confidence interval
cox1	Cytochrome c oxidase subunit 1
De.	*Dermacentor*
DNA	Deoxyribonucleic acid
dNTPs	Desoxynucleosidtriphosphate
E.	*Ehrlichia*
EDTA	Ethylenediaminetetraacetic acid
F	Female
FAO	Food and Agriculture Organization
FATA	Federally Administrated Tribal Areas
FCT	Federal Capital Territory
FAnGR	Farm animal genetic resources
GADM	Database of Global Administrative Areas
GDP	Gross Domestic Product
GIS	Geographic Information System
Ha.	*Haemaphysalis*
Hy.	*Hyalomma*
ILCA	International Livestock Centre for Africa

Abbreviations

IPM	Integrated pest management
ITS2	Internal transcribed spacer 2
Ix.	*Ixodes*
KPK	Khyber Pakhtunkhwa
L	Larva
LDDDP	Livestock and Dairy Development Department, Punjab
min	Minute
µl	Microlitre
M	Male
m	Meter
mM	Millimolar
mPCR	Multiplex polymerase chain reaction
N	Nymph
NDMA	National Disaster Management Authority
OIE	Office International des Epizooties, World Organisation for Animal Health
OR	Odds ratio
P	Probability value
PCR	Polymerase chain reaction
PLPS	Pastoral Livestock Production System
PTDC	Pakistan Tourism Development Corporation
RBCs	Red blood cells
R.	*Rickettsia*
Rh.	*Rhipicephalus*
RH	Relative Humidity
RLB	Reverse Line Blot Hybridization
rRNA	Ribosomal ribonucleic acid
s	Seconds
SDS	Sodium dodecyl sulfate
SSPE	Saline-Sodium Phosphate-EDTA
T.	*Theileria*
TBDs	Tick-borne diseases
temp	Temperature
TBE	Tick-borne encephalitis
UNESCO	United Nations Educational, Scientific and Cultural Organization
USCB	United States Census Bureau
w/v	Weight/Volume
WHO	World Health Organization
χ^2	Chi- squared

1 Introduction

1.1 Facts about Pakistan

The official name of the country is *Islamic Republic of Pakistan*. It is located in South Asia between 23° 35 minutes to 37° five minutes North latitude and 60° 50 minutes to 77° 50 minutes East longitude (AIPS, 2015). It is the sixth most populous country with a population around 200 million people (USCB, 2015). With a total area of 796,095 km^2, it is the 36th largest country in the world. It has a long coastal line (1,046 km) along the Arabian Sea in the South and is bordered by Iran to the Southwest, Afghanistan to the Northwest, China to the northeast and India to the east. In the Northeast, the disputed territory of Jammu and Kashmir is situated (Figure 1.1). The "provinces" represent the 1st administrative levesl of the country. They are five in number, namely Baluchistan, Khyber Pakhtunkhwa, Gilgit-Baltistan, Punjab, and Sindh. In addition to the provinces, there are Federally Administrated Tribal Areas (FATA) and Azad Kashmir (Figure 1.2). The "divisions" represent the 2nd administrative level. They are further partitioned into districts and tehsils. The official language of the country is Urdu.

Figure 1.1 Map of Pakistan showing its location on the continent of Asia

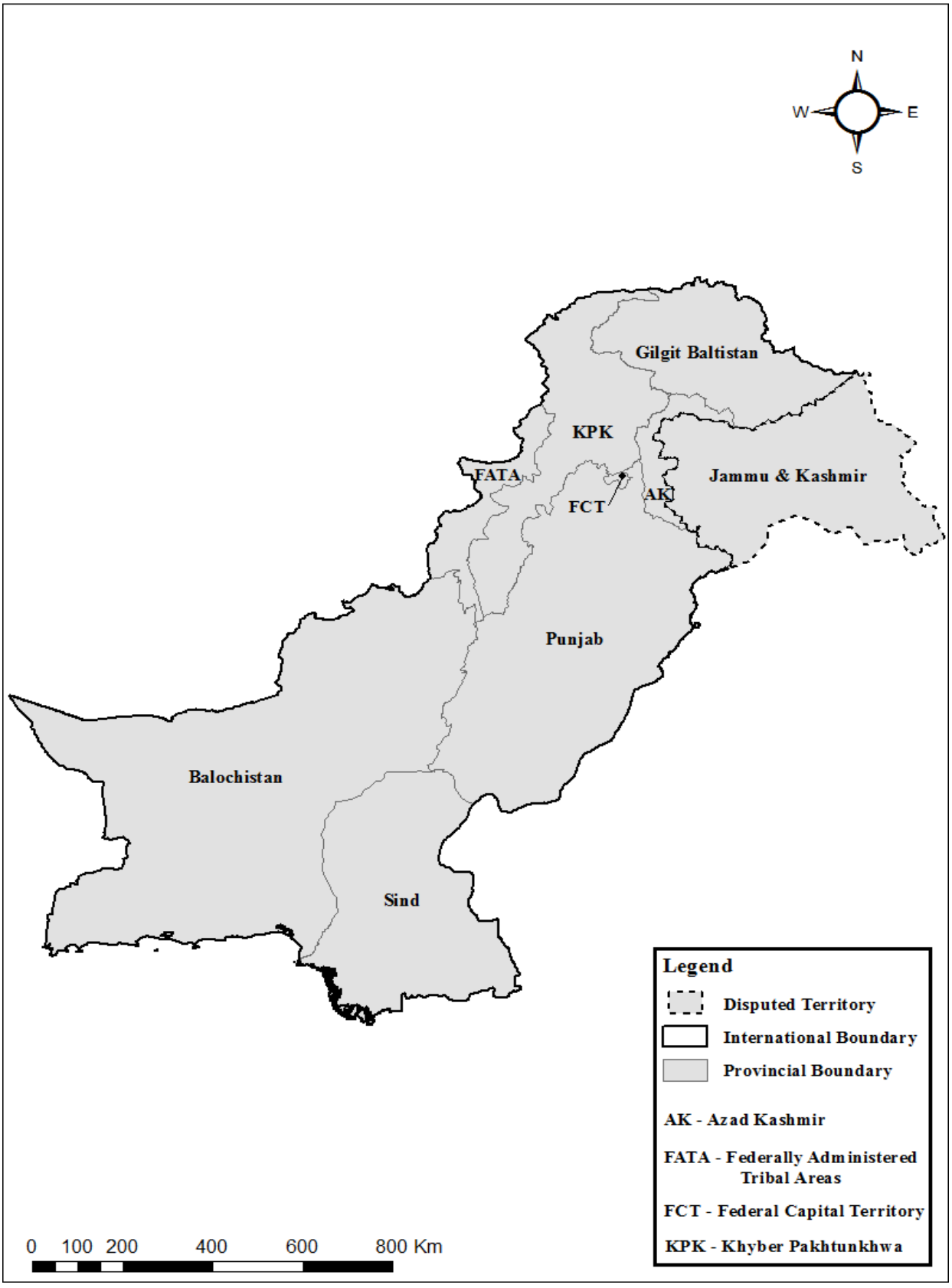


Figure 1.2 Map of Pakistan showing its provinces and federally administrative areas

1.2 Geography and climate

Pakistan has a distinct landscape. Geographically, it is divided into three regions: the Indus River plain, the Balochistan Plateau, and the northern highlands. The latter includes portions of long mountain ranges, which are comprised of Himalayan, Hindu Kush, and the Karakoram. The world's second highest peak, K2 (8,611 m), is also present in the northern highlands (PTDC, 2015). Inter-mountain valleys make up most of the KPK Province and rocky mesas cover much of the Balochistan Plateau in the west. Southwards there is the fertile Indus Plain which is alongside the Indus river. The Indus river is 1609 km long and irrigates, along with its tributaries (Chenab, Jhelum, and Ravi), the most parts of the country from the Kashmir to the Arabian Sea. The irrigated plains that lie along the Indus river cover much of Punjab and Sindh (Anonymous, 2015). Both provinces have desert areas as well: Cholistan and Thal in Punjab and Tharparkar in Sindh (NDMA, 2010).

Pakistan is divided into five major agro-ecological zones on the basis of aridity[1] (Figure 1.3) and 10 different agro-ecological zones on the basis of physiography (Khan, 2004). The climate is generally dry and most areas receive less than 250 mm of rain annually. There is a noticeable climatic difference between northern and southern regions. The mean annual temperature is around 27°C. However, temperatures vary with elevation from −10°C during the coldest months in the mountains and northern areas to 50°C in the warmest months in parts of Baluchistan, Punjab and Sindh (NDMA, 2010).

Pakistan has four weather seasons. December to February is dry and cool, March to June is very hot, July to September is the wet monsoon season, and October to November is the retreating monsoon season with high temperatures countrywide (NDMA, 2010)[2].

[1] Consultative Group for International Agricultural Research (CGIAR) using the UNESCO aridity index (AI)
[2] https://en.wikipedia.org/wiki/Climate_of_Pakistan

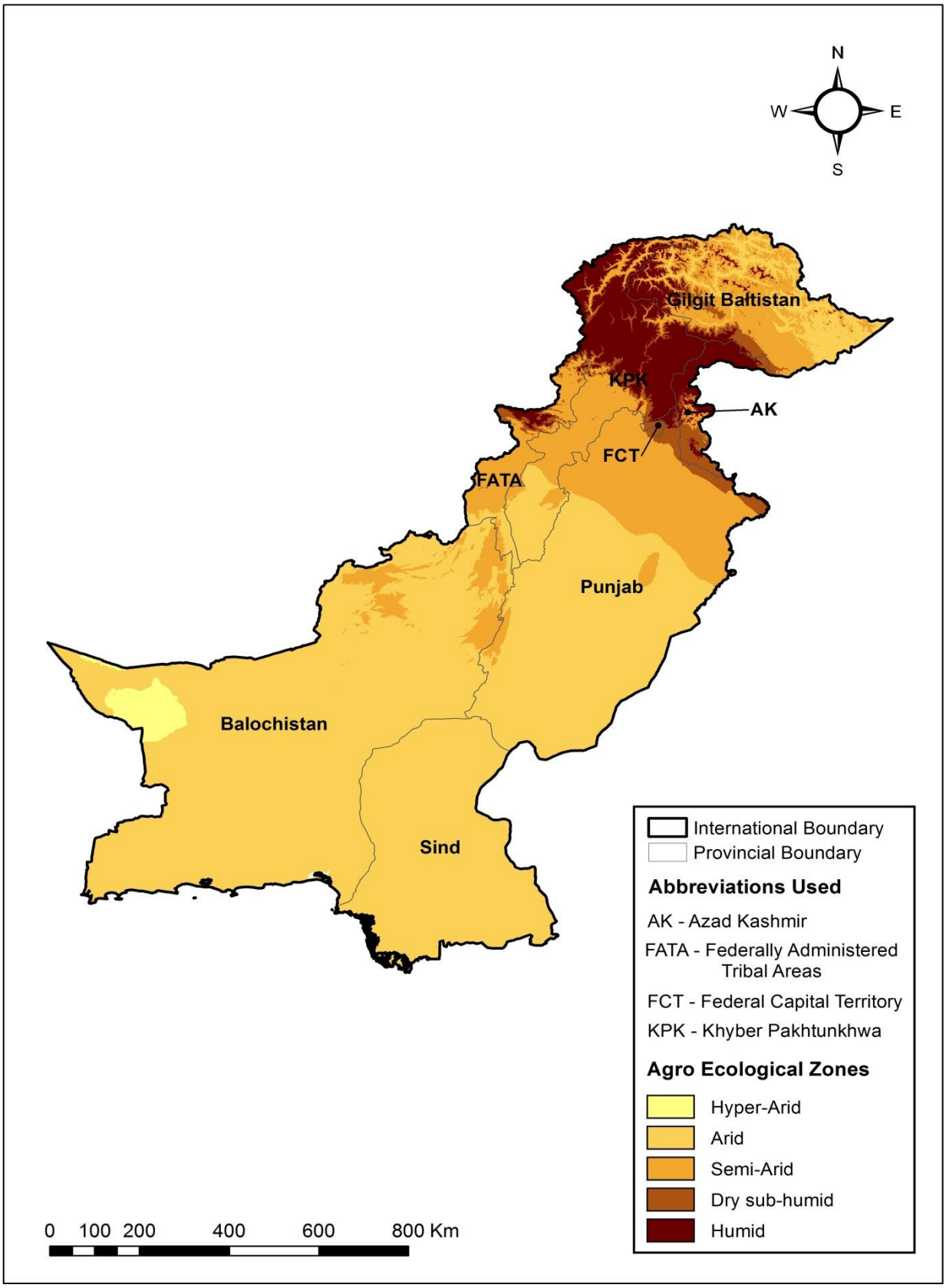


Figure 1.3 Map of Pakistan showing its administrative areas and agro-ecological zones

1.3 Livestock sector in Pakistan

Pakistan is basically an agricultural country with 21.2% contribution from agriculture sector to Gross Domestic Production (GDP). The agricultural sector is believed to be the backbone of the rural economy as it provides employment to 45% of the workforce of the country. In Pakistan, more than 70% of the population lives in rural areas and the majority depends on livestock keeping for their subsistence (Mather and Abdullah, 2015). A variety of domesticated farm animal genetic resources (FAnGR) are present, generally referred as livestock, such as buffalo, cattle, goat, sheep, poultry, camel, horses, and donkeys (Khan, 2004). Livestock is an integral part of the agriculture sector. In the financial year 2014/15 it contributed 56.3% to the agricultural sector, and 11.8% to the national GDP (LDDDP, 2015). An increase of 2.7% in Gross Value Addition of livestock has been observed in the year 2013/14 (Ministry of Finance, 2014). According to the Ministry of National Food Security and Research, the estimated cattle population is 41.2 million and there are 35.6 million buffaloes, 68.4 million goats and 29.4 million sheep (LDDDP, 2015)[3]. Buffaloes (*Bubalus bubalis*) are raised primarily in the northern and southern irrigated plains which are situated in arid and semi-arid zones. Cattle (*Bos indicus and Bos taurus*) and goats (*Capra aegagrus hircus*) are raised throughout the country especially in areas with forage and grazing facilities. A major part of the sheep (*Ovis aries*) population is reared in the western and northern hilly areas (Afzal, 2015). In Pakistan, the agro-livestock production system (ALPS) is most common, which prevails in irrigated and non-irrigated regions of the country. Small ruminants are commonly raised under the pastoral livestock production system (PLPS). In addition to ALPS and PLPS, the agro-pastoral livestock production system (APLPS), in which all the ruminant species are reared, is also present in some parts of the country (Khan, 2004).

1.4 Dairy sector in Pakistan

Almost 97% of the total buffalo population in the world (172 million heads) is raised in Asia, mainly in India and Pakistan (Rajput et al., 2005). In Pakistan, two buffalo breeds are predominant, namely Nili-Ravi and Kundi breeds. The former is considered the best dairy buffalo breed in the world (Ashraf et al., 2013; Zia et al., 2011). Based on location and herd size, the dairy sector in Pakistan consists of three types of producers; more than 80% small farmers producing > 50% of the total milk (smallholder subsistence and smallholder market-oriented), 14% medium-sized

[3]Estimated figures are based on inter census growth rate of Livestock Census 1996 & 2006

producers in the outskirts of cities, also known as gowalas, producing 29% of the total milk (with herd size of ~ 20 animals), 3% large-scale producers with a well organised farming system producing ~ 20% of the total milk (herd size > 40 animals with combinations of local cross breed and imported cows) (Jabbar et al., 2015; LDDDP, 2015; Wynn et al., 2006).

1.5 Tick species

Parasitic infestation is the major problem for animal health in most of the developing and underdeveloped countries of the world. Among the ectoparasites, ticks and tick-borne diseases (TBDs) cause substantial economic losses to poor-resource farming communities for their survival, especially in tropical and subtropical regions, where 80% of the world's total cattle population is raised (de Castro, 1997; Durrani et al., 2008; Jabbar et al., 2015; Jonsson, 2006). Tick infestations not only cause direct damage to the health of animals, but can also transmit a range of pathogens (bacteria, protozoa and viruses), which can lead to spontaneous abortion, and even death of the animal (Durrani et al., 2008; Rajput et al., 2005; Zulfiqar et al., 2012). More importantly, ticks can also transmit zoonotic pathogens to human beings and can produce serious threats to public health (Bowman and Nuttall, 2008; de la Fuente et al., 2008). Until now, around 900 species of ticks (Ixodidae = 700 and Argasidae = 200) have been recognized as valid species (Guglielmone, 2010).

1.6 Tick-borne pathogens (TBPs)

Tick-borne pathogens are mostly prevalent in tropical and subtropical regions of the world including Pakistan (Jongejan and Uilenberg, 2004; Khan et al., 2004). During the last few years, many zoonotic viral diseases have emerged in Southeast Asia (Chadha et al., 2006; Hsu et al., 2004). Ticks have been reported to transmit at least 38 viral species. Tick-borne viruses, e.g. Crimean-Congo haemorrhagic fever (CCHF) virus and Tick-borne encephalitis (TBE) virus, are maintained in nature primarily in ixodid tick vectors, specifically ticks of the genera *Hyalomma* and *Ixodes*, respectively (Labuda and Nuttall, 2004). The bacterial pathogens (*Anaplasma*, *Borrelia* and *Ehrlichia*) transmitted by tick vectors can cause a variety of diseases ranging from chronic diseases, like Lyme borreliosis, to life-threatening infections, such as tularemia (de la Fuente et al., 2008). The transmission of protozoa (*Babesia* and *Theileria*) is thought to be strictly associated with ticks (de la Fuente et al., 2008). Mainly four tick-borne diseases (TBDs), namely: anaplasmosis, babesiosis, cowdriosis and theileriosis are common in bovines all over the world

(Jabbar et al., 2015). Babesiosis and theileriosis can badly affect animal health and cause huge economic losses in the tropics and subtropics (Jongejan and Uilenberg, 2004).

1.7 Risk factors associated with tick infestation and TBDs

Epidemiological tracing and analytical investigations have revealed several risk factors for the occurrence of ticks and transmission of TBDs globally, as illustrated by studies conducted in Brazil (Labruna et al., 2001), Sudan (Salih et al., 2007), United Kingdom (Smith et al., 2011), United States of America (Eisen et al., 2013), Uganda (Muhanguzi et al., 2014) and Ethiopia (Taye et al., 2015). The effect of environmental factors such as climate (Estrada-Pena, 2009; Gilbert, 2010; Olwoch et al., 2007; Randolph, 2008) and habitat type (Fyumagwa et al., 2007; Thamm et al., 2009) on tick distribution patterns and TBDs have been investigated in different parts of the world. Similarly, the effect of host characteristics has conferred various degrees of resistance to tick infestation (Berman, 2011; Carvalho et al., 2011; O'Neill et al., 2010). Studies on risk factors for acaricide resistance in ticks have shown that a higher rate of acaricide application is associated with selection of resistant tick species (Jonsson et al., 2000; Rodríguez-Vivas et al., 2006; Spickett and Fivaz, 1992). However, the timing of treatments within the yearly cycle(s) of tick development is likely to be more important than frequency alone (Sutherst and Comins, 1979). Husbandry practices, locations, and age of the host have been found as important risk factors associated with *Theileria annulata* (*T. annulata*) infection in the Sudan (Salih et al., 2007). The abundance and distribution of ticks and presence of a susceptible host are the most important determinants associated with TBDs (Bakheit and Latif, 2002; Tatchell and Easton, 1986).

1.8 Tick species in Pakistan

Most parts of Pakistan offer favourable environmental conditions for tick multiplication. Therefore, ectoparasites are potential hazards for livestock, leading to massive economic losses through lowered productivity and mortality (Ghosh et al., 2007). Additionally, due to global warming, the environmental conditions are continuously changing and may thus alter the distribution patterns and vector potential of tick species. The most commonly reported tick species from different regions of Pakistan are *Hyalomma anatolicum* (*Hy. anatolicum*), *Rhipicephalus* (*Boophilus*) *microplus* (*Rh. microplus*), *Rh. sanguineus, Rh. annulatus* and *Hy. marginatum* (Durrani et al., 2008; Ramzan et al., 2008; Sajid et al., 2008). *Hyalomma* species transmit a number of viral, bacterial and parasitic diseases in different parts of the world, making these ticks the economically most important ixodids (Mehlhorn, 2012). *Rh. microplus*, also known as the cattle

tick is considered to be the world's most important tick parasite of livestock (Anonymous, 2007; Levin, 2015a).

1.9 Tick-borne pathogens in Pakistan

Theileriosis (typically caused by *Theileria annulata*), babesiosis (*Babesia bovis* and *B. bigemina*) and anaplasmosis (*Anaplasma marginale* and *A. centrale*) have been reported in Pakistan (Ashraf et al., 2013; Atif et al., 2013; Durrani and Kamal, 2008; Jabbar et al., 2015). Given the relatively rapid expansion of the dairy industry and the increased importation of high milk-producing dairy cattle (e.g., Holstein, and Friesian) from overseas countries to replace and/or improve indigenous local breeds of cattle, it has become crucial to assess the status of TBDs in indigenous and exotic breeds of cattle and water buffaloes, as exotic breeds are usually highly susceptible to TBDs (Darghouth et al., 2010).

Almost all of previous studies conducted in Pakistan used a conventional diagnostic method (stained blood smear method) to estimate the prevalence of TBDs, which highly depends on skilled laboratory staff and where inter-observer bias could affect the results severely. However, only a few studies utilized PCR for the detection of TBDs (Jabbar et al., 2015). Recently, the development of new molecular techniques like multiplex polymerase chain reaction (mPCR) and use of simple PCR in a reverse line blot (RLB) hybridization framework have allowed the simultaneous detection of different pathogens with very high sensitivity and specificity and could provide additional epidemiological information (Ahmed et al., 2002; Schnittger et al., 2004).

1.10 Investigation of risk factors in Pakistan

So far, many aspects of the epidemiology of ticks and TBDs in Pakistan are unknown. To allay the risk of spread of TBDs, it is necessary to devise an evidence-based tick control programme for the livestock sector. To date, only a few studies have investigated the risk factors associated with high tick infestation on livestock farms in Pakistan (A. Iqbal et al., 2013; Sajid et al., 2009). Non-cemented floor, grazing and tethering of animals were found associated with high tick infestation in ruminants (A. Iqbal et al., 2013; Sajid et al., 2009). Similarly, the risk factors associated with TBDs have also been studied rarely. Anaplasmosis in buffaloes was found to be positively associated with the presence of dogs on livestock farms (Ashraf et al., 2013).

1.11 Rationale of the study

Although the climatic conditions of Pakistan are suitable for the rapid growth of various tick species, there still is a lack of systematic work to investigate frequency and distribution of tick species infesting ruminants (Durrani et al., 2008). Many of the previously conducted studies were confined to a small area and did not consider agro-ecological zones, production systems and sampling strategies, which are all factors that can affect prevalence estimates of ticks and TBDs (Jabbar et al., 2015). Many of the previous studies estimated tick prevalence, but they selected those animals that were known to be infested. Moreover, to date, there is no study from Pakistan that confirmed the identified tick species using molecular methods like sequencing which could produce a different result, as the microscopic identification is subjective and heavily depends on the expertise of the investigator. It is crucial to obtain correct and precise information and to map the existing prevalence and distributions of ticks and tick-borne pathogens, otherwise, changes in distribution and prevalence patterns cannot be tracked.

Although a number of studies have been conducted to estimate the prevalence of tick-borne pathogens in farm animals and tick vectors in Pakistan (Ali et al., 2013; Atif et al., 2012; Durrani and Kamal, 2008; M. K. Khan et al., 2013), almost all of them used conventional methods as diagnostic tools for TBDs. Therefore, the paucity of accurate data on the epidemiology of ticks and TBDs makes it difficult to estimate the economic losses rendered by tick infestation and TBDs. There are many risk factors associated with tick infestation in farm animals, which in turn has a direct impact on the epidemiology of TBDs (Tatchell and Easton, 1986). Therefore, identification of these risk factors could contribute a vital role in designing cost-effective tick control measures.

Taking into account the lack of information regarding the epidemiology of ticks and tick-borne pathogens, the aim of this study, therefore, was (i) to estimate the tick prevalence among farm animals in Punjab province, (ii) to identify and confirm the tick species infesting farm animals through molecular studies, (iii) to find out different tick-borne pathogens circulating in the study area, (iv) to assess the percentage of infection in the tick species regarding these pathogens, and (v) to find out potential risk factors associated with high tick infestation that could facilitate the development of effective interventions to control the problem.

2 Review of literature

2.1 Ticks

Ticks are invertebrate animals in the order Acarina and sub-order Ixodida (Anderson, 2002). The study of ticks, along with mites, is known as acarology. Ticks are blood feeding ectoparasites of mammals, birds, and occasionally reptiles and amphibians (only one tick species). They transmit a number of microorganisms, which cause diseases in humans and animals (Murrell and Barker, 2001).

2.2 Taxonomic classification

Ticks belong to 896 species, which are grouped in three families, namely Argasidae, Ixodidae and Nuttalliellidae, of which the first two are important to domestic animals (Guglielmone, 2010; Sonenshine and Roe, 2014).

The Ixodidae is the most important and cosmopolitan tick family, which is comprised of 702 species in 14 genera (Guglielmone, 2010). They are commonly known as 'hard ticks' because, unlike Argasidae, they have a hard dorsal shield (scutum). If the tick is un-engorged, the shield normally can bear the force of a human's footwear. Unlike Argasidae, they attach to their host for longer duration (Lowchens of Australia, 2016). Another distinct feature in nymph and adult stages of the Ixodidae, is a prominent head (also known as capitulum), which projects forwards from the body (Molyneux, 1993).

The members of Argasidae family are commonly known as 'soft ticks'. The family includes 193 species, but there is widespread disagreement concerning the genera in this family and the proper composition of the genus is under review (Guglielmone, 2010). With a few exceptions, majority of them primarily feed on birds. Unlike hard ticks, they feed rapidly (in minutes), and the bites are really painful. They live in animal surroundings, such as in animals' nests, crevices or burrows, whereas hard ticks mostly stay on the host (Allan, 2001).

The family Nuttalliellidae contains only one species, which has only been found in Tanzania and South Africa (Evans, 1992; Guglielmone, 2010). It can be differentiated from the other two families by some distinct features, such as the position of the stigmata, lack of setae, and intensely grooved integument (Roshdy et al., 1983).

2.3 Geographic distribution

Like other arthropods, ticks are quite sensitive to climatic changes as they spend most of their time in the environment (Estrada-Peña et al., 2012). Dynamics of tick population is controlled by many factors including host availability, vegetation and climatic variables (Wall and David, 2001). The latter is considered perhaps the most important driver to the absence or presence of a tick species in a specific zone (Cumming, 2002), as they are heavily affected by changing the environmental temperature and climatic conditions (Randolph, 2010, 2004). However, climate factors affect tick survival particularly during free-living state of their life cycle (Ogden et al., 2004).

Although ticks are widely distributed all over the world, they have a tendency to prosper more in warm and humid climates, because they need a specific level of humidity in the air for the process of metamorphosis (Magnarelli, 2009). It has been observed that low temperatures prolong the developmental cycle of tick species and subsequently increase the tick mortality rate. On the other hand, warm climate conditions contribute to faster development and hence lower mortality, which increases the probability to adapt a new environment (Ogden et al., 2004). Tick species of domestic animals are exclusively common in tropical regions, where they cause significant economic losses.

Tick species, particularly *Ixodes scapularis* (causative agent of Lyme disease), have been investigated using geographic information systems (GIS) to explore the dynamics of tick populations and to build predictive models for ultimate tick habitats. In previous studies, the presence of particular environmental features, such as hardwood trees, rivers, sandy soil, and host availability, were revealed to be reliable predictors of higher tick densities (Allan, 2001).

2.4 Diet and feeding behaviors

Ticks are obligate blood-feeding ectoparasites of domestic and wild animals and humans. They need a meal of blood at each stage of development (CDC, 2013a). Ticks are unable to fly or jump on their hosts, but many tick species find their hosts through a behavior called "questing". While questing, the tick leaves the ground level, climbs up and holds onto vegetation by their third and fourth pair of legs, extends the first pair of legs and adopts a sit-and-wait tactic for finding a suitable host (Tomkins et al., 2014; Wilson, 2016). The environmental conditions that influence questing behavior have been widely explored, and temperature is considered the most important factor (Perret, 2008; Perret et al., 2004). Ticks can detect the breath of animals (CO_2) as they exhale and body odors, sense body heat and moisture. Tick species that have eyes can sometimes even

detect color and movement of the host (CDC, 2015; Wilson, 2016). As a host encounters the spot where a tick is waiting, mostly it quickly attaches to the host, but some tick species look for areas where skin is thinner (CDC, 2013a). After finding a suitable feeding spot, the tick grabs the host's skin and cuts into it (CDC, 2013a). Ticks use sharp chelicerae to make a hole into the dermis and secrete cement (an adhesive material), which assist them in attachment, also by mounting the hypostome using the secreted cement in the hole prepared in the dermis. They also secrete some anticoagulant substances in their saliva, which stops the blood from clotting and minimize the inflammatory reactions. These mechanisms altogether help them in sucking blood from their host (Goddard, 2008). Although they rely on blood as their only food source and need a blood meal at each developmental stage, they do not only survive but also flourish (de La Fuente et al., 2015). Ticks are highly adapted for long-term survival off the host without feeding and can extract moisture directly from humid air. However, survival is greatly reduced by excess heat, dryness, and lack of suitable hosts. Survival on the host is also greatly reduced by grooming and by hypersensitive immune reactions in the skin against tick feeding (Sonenshine and Roe, 2014).

2.5 Life cycle

All the tick species belonging to the Ixodidae or Argasidae families undergo three developmental stages: larval, nymphal, and adult (Munderloh et al., 2005). Most of them follow one of four different life cycles (CDC, 2013a).

2.5.1 Ixodidae

All the members of the Ixodidae undergo either one-host, two-host or three-host life cycles. A fully engorged female can lay 5,000 to 10,000 eggs on the ground and from these eggs larvae emerge (de La Fuente et al., 2015). These larvae then wait for or attach to their hosts, primarily small mammals and birds, for feeding. After a blood meal, larvae remain on the same host for the next two (nymphal and adult) stages, only leaving the host prior to laying eggs (one-host life cycle) or they moult to nymphs on the same host, but leave the host before adult stages (two-host life cycle) or they detach from their host and moult to nymphs on the ground (CDC, 2013a). The nymphs then wait on the vegetation and take on the "quest" until they end up on a host where they feed. After blood meal, they detach from their host and fall down to the ground where they digest the blood meal and moult to adults, either a female or a male (Sonenshine and Roe, 2014). The adults wait for their host and get attached to it (three-host life cycle). During the two-host life cycle, the second host may be the same individual, another individual from the same species, or even from a

different species (CDC, 2013a). Most members of the Ixodidae (625 out of 650 species) require three hosts to complete their life cycle, which takes at least one year. These three hosts are not always from the same species, but may be the same species, or even the same individual, depending on host availability for the tick (CDC, 2013a). Female ticks spend less time on their host, as after feeding they have to detach to lay eggs on the ground, while males stay for longer time mainly for mating purpose but they suck little amount of blood as compared to females (Allan, 2001).

2.5.2 Argasidae

Most species of the family Argasidae follow a multi-host life cycle. The female members of the family can lay a few hundred to over a thousand eggs during her complete life span. Larvae feed on host (from 1 hour to several days, depending on tick species) and detach themselves to moult to nymphs. Argasid ticks, unlike ixodid ticks, may take two or more nymphal stages (known as instars), each requiring a blood meal. Nymph also leave the host and moult to adults in the shelter area. Adult ticks quest for a host and after blood sucking they detach from the host. Unlike ixodids, mating is done off the host. Nymphs and adults of the Argasidae are adapted to feeding quickly (about an hour) as compared to ixodid ticks, which stay several days on their hosts (CDC, 2013a). Their life cycles range from months to years depending on the environmental conditions. During feeding, they excrete large amounts of fluid from the coxal glands which makes them unique as compared to the other two families (Allan, 2001).

2.6 Important ticks of domestic animals

2.6.1 *Boophilus* species

The genus has been revised and retained as a subgenus of *Rhipicephalus* and the name "*Boophilus*" has been synonymized with *Rhipicephalus* (sensu lato) (including *Boophilus*) (Murrell and Barker, 2003). The members of the genus have a global impact on the livestock industry by causing direct (parasitic) and indirect (transmission of microbes) losses. In addition, *Rhipicephalus (Boophilus) microplus*, which is also known as cattle tick, is considered to be the most important tick species for the livestock industry (Anonymous, 2007; Levin, 2015a). Other members, such as *Rh. annulatus*, which is ubiquitous in tropical and subtropical areas, and *Rh. decoloratus,* which is found in Africa, also play a key role in the transmission of tick-borne pathogens to domestic animals. A number of studies from Pakistan have reported *Rh. microplus* from central and northern part of the country (A. Iqbal et al., 2013; Irshad et al., 2010; Mustafa et al., 2014).

These ticks are adapted to feed on a variety of domestic animal species including cattle, buffaloes, goats, sheep, horses, donkeys, deer, pigs, dogs and some wild animals. They undergo a single-host lifecycle and all the feeding stages occur on same individual in a rapid progression (Sonenshine and Roe, 2014). Infestation starts when a larva on the vegetation attaches to a host. The moults are quite rapid and the subsequent stage stays to commence feeding again. The collective feeding and moulting periods last approximately 21 days. The engorged female leaves the host and lays a single batch of eggs comprising of 2000 eggs. They can also survive by feeding on some wild bovid hosts. It is generally assumed that *Rh. microplus* had been introduced from the Indian forests to the other parts of the world (Levin, 2015a).

2.6.2 *Hyalomma* species

These are often the most abundant parasites of domestic animals in southern part of Africa and Europe and central and southwest Asia. The genus includes many important tick species of Ixodidae. It has been reported as the most common tick genus in Pakistan in recent years (Ali et al., 2013; Iqbal et al., 2014; Sajid et al., 2011). The most common species include *Hy. anatolicum*, *Hy. marginatum rufipes*, *Hy. truncatum*, *Hy. detritum detritum*, and *Hy. dromedarii*. The former four species feed on domestic ruminants, whereas the latter is adapted to feed on camels. Members of the *Hyalomma* spp. can survive in territories with a wide temperature range and very low precipitation through diapause, which help them in adjusting to these conditions. Another adaptation is that the same tick species can undergo a two-host or a three-host life cycle. For instance, in a two host lifecycle: *Hy. anatolicum* may complete larval and nymphal stages on a hare and adult stage on a cow, or in a three-host cycle it may feed as a larva on a hare, then as a nymph on gerbil and then as an adult on cow. Nevertheless, this tick species often follows a three-host cycle.

2.6.3 *Rhipicephalus* species

They are distributed globally throughout the warm countries and have been reported from all continents except Antarctica (Bowman, 2011; Nava et al., 2015; Szabó et al., 2005). The members of this genus are proven vectors of pathogens of medical and veterinary importance (Bowman, 2011; Otranto et al., 2009; Parola et al., 2005). In spite of their huge significance, taxonomic classification and orderly relationship among species are still controversial (de Oliveira et al., 2005; Nava et al., 2015; Pegram et al., 1987; Zahler et al., 1997). Typical *Rhipicephalus* species that feed on cattle are *Rh. appendiculatus*, the brown ear-tick, and *Rh. evertsi evertsi*, the red-

legged tick. These ticks follow a three-host lifecycle, where the female feeds only on a particular animal species that is obligatory for the completion of life-cycle. The female takes a heavy blood meal and leaves the host for egg laying. The female lays a single batch of about 20,000 eggs and dies afterwards. The eggs moult into larvae on the ground and larvae quest for the animal host. After finding a suitable host, they quickly attach to the host and start feeding, which continues for about 5 days. After a successful blood meal, the larva detaches from the host and moults into a nymph, which then waits for another host of a different animal species. The nymph feeds for 5 to 10 days and leaves the host to moult into the adult tick and the cycle continues. The total cycle takes about 8 to 20 months depending upon diapause in adaptation to the climate.

2.6.4 *Amblyomma* species

The genus is comprised of 129 species that are characterized by long mouthparts and beautiful ornamentation on the scuta. These three-host ticks are commonly present in tropical and subtropical countries where they feed on domestic and wild animals including birds (Jongejan and Uilenberg, 2004). Birds may play a crucial part in the dispersion of *Amblyomma* ticks. The most important *Amblyomma* species are *Am. variegatum* and *Am. hebraeum*. These species have widest distribution, particularly on the African continent, and are well adapted to feed on domestic livestock (Jongejan and Uilenberg, 2004).

2.6.5 Other genera of ticks

Other important tick genera of domestic animals include *Dermacentor andersoni* (commonly known as the Rocky Mountain wood tick), *De. variabilis* (the American dog tick), *De. reticulatus*, (the ornate dog tick of Europe); *Haemaphysalis leachii* (the yellow dog tick of the tropics); *Ixodes ricinus* (the deer tick of Europe), *Ix. scapularis* (the black-legged tick of North America), *Ix. holocyclus* (the paralysis tick of Australia).

Table 2.1 Key identification features of important genera of ticks of domestic animals

Family	Genera	Size	Mouthparts	Other features
Argasidae (Soft ticks)	*Argas, Ornithodoros, Otobius*	Large	Ventral and short	Scutum and pulvilli absent
Ixodidae (Hard ticks)	*Amblyomma, Hyalomma*	Large	Anterior and long	Pale rings on legs Eyes present and large
	Ixodes	Medium	Anterior and long	Plain dark legs Eyes absent

	Dermacentor, Rhipicephalus	Medium	Anterior and short	Eyes present and large Coxae 1 with paired spurs
	Boophilus, Haemaphysalis	Small	Anterior and short	Eyes small or absent Coxae 1 with small paired spurs or single spur

2.7 Collection of ticks

Ticks can be collected either from the environment/vegetation or directly from the bodies of animals depending on the objective of the study. A majority of the researchers, who focused on tick-borne diseases, whether human or animal, as their primary objective, collected ticks directly from the animals (Aktas et al., 2012; Al-Khalifa et al., 2007; Durrani et al., 2008; Hilpertshauser et al., 2006; Mourya et al., 2012; Ramzan et al., 2008; Steyn et al., 2010), while many researchers, who studied only the distribution and ecology of ticks, have collected tick samples, using dragging or flagging methods, from forests or vegetationthat were separate areas and not the part of surroundings of the animals (Gherman et al., 2012; Terassini et al., 2010).

Tick collection methods had been reviewed by Gray (1985). He divided these methods into four major categories: (1) flagging or dragging methods; (2) trapping using carbon dioxide baits; (3) collecting from hosts and (4) walking through forest, i.e. on the clothes of the collectors. Different researchers considered different predilection sites including ears, brisket, withers, legs, udder, perineum and tail regions for collection of ticks to get the real picture.

Among the flag methods, two types of flags can be used; 1) CO_2 flagging and 2) CO_2-free flagging. A study used both kinds of flags and compared the results, which showed a significant increase in collection of *Ixodes ricinus* ticks using CO_2-enhanced flags but the results of the study could not be extrapolated for other tick species as the authors mentioned in the article (Gherman et al., 2012).

2.8 Tick counting methods

Various procedures have been employed to estimate the total tick burden on an animal in the past and among those patch sampling is rapidly becoming very popular method. Patch sampling has been suggested for obtaining measures of relative tick burden rather than absolute tick counts and is comparatively quick and easy to perform in a field situation (Mooring and McKenzie, 1995). Patch sampling might also represent a reasonable sampling method for obtaining measures of relative tick load on animals in different geographic areas, between members of different species or as a measure of seasonal variation in tick burden.

There is also the method of targeted counting of ticks at their predilection sites on live animal (Londt et al., 1979). Work by Baker & Ducasse (1967) on the predilection sites of cattle ticks suggested that tick numbers sampled from small areas on the predilection sites might be used to compare the size of tick populations quantitatively (Rechav, 1982). In practice, MacLeod & Colbo (1976) patch sampled an area of 100 cm^2 on seven predilection sites of cattle, Kaiser et al. (1982) collected from eight sites on one side of the body surface for cattle and on both sides for sheep, while Kaiser et al. (1991) collected only from the ears of cattle. The basis for patch sampling is the well-known fact that ticks tend to concentrate on certain predilection sites of the host body surface (Baker and Ducasse, 1967; Howell et al., 1978; Kaiser et al., 1982; Sachs and Debbie, 1969).

2.9 Preservation of ticks

Various tick preservation methods have been used by researchers in the past. A few of them preserved ticks, after washing them with sterile water and 70% ethanol, at −80 ºC (Aktas et al., 2012; Sang et al., 2006), while others preserved them in 70% ethanol at 4 ºC (Hilpertshauser et al., 2006). A comparative study focused on quality and quantity of DNA extracted from ticks, which were preserved using different methods, found that killing ticks in 70% ethanol and keeping them in a refrigerator at 4 ºC was the best preservation method (Mtambo et al., 2006). However, the focus of the study was to check the quality of extracted tick DNA and not the DNA of pathogens (virus, bacteria or protozoa) that might be present in ticks. Thus, the results could not be equally applied to investigations of tick-borne pathogens. The University of North Florida (UNF), Public Health Research Laboratory, has recommended 70% isopropyl alcohol or slightly higher concentrations for the preservation of ticks. In contrast to this, Cruickshank (2002) found that the most effective method to preserve insects for DNA extraction was ultra-cold (−80 ºC) freezing of live specimens and the second most effective method was killing and preserving them in 100% ethanol at 4 ºC. In a study conducted in Saudi Arabia, live ticks were transported from the study area to the Virology Division of Naval Medical Research in Cairo, Egypt, where the samples were frozen at −70 ºC (Al-Khalifa et al., 2007).

Ticks can also be transported alive from the field to the laboratory by placing a moist paper in the transport vessel, but not for a longer period. Steyn et al. (2010) transported ticks from field to the laboratory for molecular studies by storing them in a screw-caped plastic container, with holes in the lid, inside a larger zip-lock plastic bag kept in a cooler box with an ice brick. DNA is a relatively

stable molecule that may stay intact for an extremely long time under appropriate conditions. The time span, over which DNA integrity of biological specimens is maintained, is a function of the preservation conditions (Phillips and Simon, 1995).

2.10 Current knowledge of tick species in Pakistan

Several studies have been conducted to describe the distribution of tick species in different regions of Pakistan, which have been summarized in Table 2.2. These studies were only based on microscopic examinations and none of them confirmed their findings through molecular techniques.

Table 2.2 Studies conducted to investigate the distribution of tick species and their prevalence in different districts of Pakistan

Study area	Host	Tick species	Study duration	No. Ticks collected	Prevalence	Reference
Attock, Islamabad	Goat Sheep	*Rhipicephalus* *Haemaphysalis* *Ixodes* *Amblyomma*	Feb to Jul & Oct to Nov 2009	NP	41.53% 43.37%	(Irshad et al., 2010)
Charsadda, Swabi	Cattle	*Hyalomma* *Boophilus* *Haemaphysalis* *Rhipicephalus*	2010 to 2011	100	NP	(Ahmad et al., 2014)
Faislabad, Jhang, Khanewal	Buffalo Cattle	*Hyalomma* *Boophilus* *Amblyomma* *Haemaphysalis*	Jul to Aug, 2007	6263	34% 70%	(Ali et al., 2013)
Kasur	Cattle	*Hyalomma* *Boophilus* *Haemaphysalis* *Rhipicephalus*	Information missing	100	NP	(Durrani and Kamal, 2008)
KPK, FATA	Buffalo Cattle	NP	Jul to Sep, 2013	NP	22.58% 33.36%	(A. Khan et al., 2013)
Lahore, Multan, Rawalpindi	Cattle	*Hyalomma* *Boophilus* *Haemaphysalis* *Rhipicephalus*	Information missing	300*	NP	(Durrani et al., 2008)
Lahore	Sheep	*Hyalomma* *Rhipicephalus* *Boophilus*	Spring & summer 2007	100	NP	(Durrani et al., 2011)
Layyah, Muzaffargarh	Buffalo Cattle Goat Sheep Camel	*Hy. anatolicum* *Rh. sanguineus*	Information missing	NP	40.08% 75.1% 51.6% 0% 0%	(Sajid et al., 2008)
Layyah, Muzaffargarh	Buffalo Cattle	*Hy. anatolicum* *Rh. sanguineus*	Jul, 2006 to Jun, 2007	NP	47.3% 72.9%	(Sajid et al., 2009)
Layyah, Muzaffargarh	Goat	*Hy. anatolicum* *Rh. sanguineus*	Jul, 2007 to Jun, 2008	NP	60.1%	(Sajid et al., 2011)
Toba Tek Singh	Buffalo	*Hy. marginatum* *Rh. microplus*	Apr, 2010 to Mar, 2011	NP	31.21%	(A. Iqbal et al., 2013)
Toba Tek Singh	Goat	*Hy. anatolicum* *Rh. microplus*	Apr, 2011 to Mar, 2012	NP	11.14%*	(Iqbal et al., 2014)
Multan	Buffalo	*Hyalomma* *Boophilus* *Haemaphysalis* *Rhipicephalus*	Sep, 2009 to Aug, 2010	NP	52.5%	(Tasawar et al., 2014)

*Including other ectoparasites (lice and mites), NP = Not provided, KPK = Khyber Pakhtunkhwa, FATA = Federally Administered Tribal Areas

In animals, tick infestations are much more severe than in humans. Animals can be parasitized by hundreds or even thousands of ticks, which obviously multiplies the effect on the host, either by direct injuries or disease transmission (Bowman and Nuttall, 2008). These losses can be classified into two main groups, namely 1) Direct harms and 2) Indirect harms

2.11 Direct harms to domestic animals

These include (i) the direct effect of attachment and feeding, (ii) the injection of toxins, (iii) hide damage due to their bites, (iv) a reduction in weight gain due to the sucking of blood by adult female ticks (e.g., *Rh. microplus*), and (v) reduced milk production or quality (Biswas, 2003; Jonsson et al., 2008; McLeod and Kristjanson, 1999).

2.11.1 Biting stress and production losses

The feeding of hard ticks produce inflammation on the biting site, which triggers immune reactions against proteins present in tick saliva. This immune response is very helpful to control infectious agents, but meanwhile it produces itching (pruritus) and pain, which is known as biting stress. The sffect of this biting stress can result in reduced feed intake and anemia, which may lead to lower milk production or growth compared to hosts without tick infestation (Jonsson, 2006; Pegram et al., 1991). Tick loads on domestic and wild animals can be high, but this usually happens only in a small proportion of animals in the herd. The bite of soft ticks whilst they feed is much more painful than that of hard ticks and produces severe biting stress; a notorious example is *Ornithodoros savignyi*.

2.11.2 Tick poisoning

During feeding, ticks secrete saliva containing different types of toxins, which can cause disturbance in blood flow and may downregulate the immune response. This condition is known as toxaemia and can lead to life-threatening tick paralysis. For example, *Ix. rubicundus* causes tick paralysis in sheep in South Africa, which often ends with death of the host animal; *De. andersoni* can cause the same condition in cattle in North America and *Ix. holocyclus* may cause tick paralysis in cattle, dogs and humans in Australian (Barker and Walker, 2014; Sonenshine and Roe, 2014).

2.11.3 Physical damage

When hard ticks feed for a longer period on a host, they produce wounds, which may later lead to the formation of scars. When the animal hides are processed in the leather industry, these scars remain as blemishes, which may reduce the leather quality.

2.11.4 Other problems

These include reduced suckling by calves (caused by *Am. variegatum,* which often feed on the udder), lameness in sheep and goats (caused by *Hy. truncatum,* which feed on the feet of small ruminants), and larger wounds, which make the host susceptible to larvae of flies that can cause myiasis (Pegram et al., 1991; Stachurski, 2000).

2.12 Indirect harms to domestic animals via transmission of tick-borne diseases

The indirect losses include morbidity and mortality associated with the diseases that are transmitted by ticks. Ticks transmit more infectious agents than any other blood-feeding arthropod. They transmit a number of pathogens including viruses, bacteria, and protozoa to animals and humans (de La Fuente et al., 2015; Jongejan and Uilenberg, 2004). These pathogens may cause various diseases in the respective hosts, which are called Tick-borne diseases (TBDs). A number of TBDs (more than 16) of humans and animals (around 19) have been described in previous studies (Nicholson et al., 2009; Sonenshine and Roe, 2014). Recently, a new TBD, which is caused by *Bourbon virus,* has been reported in Kansas in 2014 (Lowes, 2014) and this trend of emerging TBD is most likely to continue. These diseases subsequently result in reduced milk, meat and wool production, may induce abortion in pregnant animals, and increase mortality in livestock herds. Infections by tick-borne pathogens not only result in economic hardship for livestock farmers, but zoonotic agents can also be transmitted to people (Bowman and Nuttall, 2008). Ticks are suitable hosts for the multiplication of many types of microorganisms and act as vectors in the transmission of these pathogens from one animal to another. They can transmit pathogens within the same animal species or across species. They have acquired a strict biological relationship with the pathogens, which they transmit (de La Fuente et al., 2015). However, some pathogens, such as *Anaplasma marginale* and *A. centrale*, do not always follow this kind of relationship and can also be transmitted by biting flies or through direct transmission, e.g. via blood transfusion. A typical feature of TBDs is the epidemiological state of endemic stability in livestock herds. This is due to increased levels of immunity against tick-borne pathogens. This immunity can be active, which is developed as a result of infection from infected ticks in early age, or passive, which is acquired from the mother by uptake of colostrum.

2.12.1 Viral diseases

Ticks can transmit many viruses, which are maintained in nature mainly in hard ticks, specifically by ticks of the genus *Hyalomma* (*Hy. truncatum, Hy. m. rufipes*, and *Hy. m. turanicum*) (Labuda

and Nuttall, 2004). Ticks of the genus *Rhipicephalus* transmit Nairobi Sheep Disease virus in East Africa. Ovine Encephalomyelitis (also known as Louping ill) is an acute tick-borne viral disease, which is transmitted by *Ix. recinus*, has been reported in sheep flocks from the United Kingdom, the Soviet Union and Czechoslovakia.

2.12.2 Bacterial diseases

Ticks can also transmit a number of important bacterial infections to domestic animals. Among those infections, anaplasmosis and ehrliciosis are most common throughout the world.

2.12.2.1 Anaplasmosis

Anaplasmosis is a disease of domestic and wild ruminants, caused by rickettsiae of the genus *Anaplasma*. It is prevalent in subtropical and tropical regions worldwide, including Asia, Africa, southern Europe, South and Central America, the USA, and Australia (Lew-Tabor, 2015). The genus *Anaplasma* includes *A. marginale*, *A. phagocytophilum*, *A. bovis* (previously known as *E. bovis*), and *A. platys* (formerly *E. platys*). These *Anaplasma* species enter into blood cells, particularly red blood cells (RBC), through the bite of an infected tick and start multiplying in their respective mammalian hosts. *A. marginale* is most prevalent tick-borne livestock pathogen globally. It is endemic in tropical and subtropical regions and is the most pathogenic species responsible for anaplasmosis (Shih, 2011). Despite this fact, still no widely accepted vaccine against *A. marginale* is available (Kocan et al., 2003). *A. centrale,* which generally causes mild disease, can also infect cattle. *A. ovis* produce mild to severe disease in small ruminants. *A. phagocytophilum* cause tick-borne fever and reproductive problems in sheep. It may infect cattle; however, it does not produce clinical disease. *A. platys* is endemic worldwide and cause coinfections with *E. canis* (Lew-Tabor, 2015).

About 20 different tick species, mainly *Hyalomma* and *Rhipicephalus* species, have been reported to transmit *Anaplasma* spp. (Camus and Uilenberg, 2010; Kocan et al., 2004). *Rhipicephalus* (*Boophilus*) spp. are major tick vectors in Africa and Australia (Lew-Tabor, 2015).

Anaplasmosis has numerous forms, ranging from mild to fatal infections, depending on the virulence of *Anaplasma* species, host susceptibility, and coinfections. Clinical signs of the diseases include fever, anaemia, jaundice, loss of appetite, dullness and depression, decreased milk production, abortion in pregnant animals, and death, mainly in exotic animals (Camus and Uilenberg, 2010). Clinically, the disease is similar to babesiosis and theileriosis. Animals that

recover from the disease remain in carrier state (Camus and Uilenberg, 2010). Bovines suffering from anaplasmosis are commonly treated with tetracyclines or imidocarb dipropionate (Camus and Uilenberg, 2010).

2.12.2.2 Ehrlichiosis

Ehrlichiosis is a tick-borne bacterial infection caused by bacteria of genus *Ehrlichia* (CDC, 2013b). Among *Ehrlichia* spp., *E. ruminantium* is responsible for causing a disease named heartwater or cowdriosis in domestic animals. It is transmitted by *Am. hebraeum* and *Am. variegatum*. *Ehrlichia* species enter into blood stream, particularly white blood cells (WBC), through bite of an infected tick and start multiplying by down regulating the host immunity against bacteria. This makes the animal also susceptible to the other bacterial infections. Clinical manifestations of the diseases include fever, flu like symptoms, neurological tremors and coughing. The name of the disease is derived after observing the prominent sign of pericardial edema. Clinical cases are generally treated with sulfonamides and tetracyclines.

2.12.3 Protozoal diseases

The transmission of protozoa (*Babesia,* and *Theileria*) is thought to be strictly associated with ticks (de la Fuente et al., 2008). Diseases caused by these tick-borne pathogens pose important problems for the health and management of domestic ruminants in the tropics and subtropics (Jongejan and Uilenberg, 2004).

2.12.3.1 Babesiosis

Babesiosis, or tick fever, is one of the most common infections of domestic and wild animals worldwide. Animal babesioses can be caused by at least 14 distinct species of *Babesia* (Riek, 1968), however, *B. bovis*, *B. bigemina*, and *B. divergens* are the most important species from an economic point of view. The disease occurs in tropical, subtropical, and temperate areas of the world and affects more than a billion cattle globally (Figueroa et al., 2010). *B. bovis* and *B. bigemina* are mainly transmitted by *Rhipicephalus* (*Bo.*) tick species (Figueroa et al., 2010). *B. bovis* is the most virulent species. It is distributed in Asia, Africa, Australia, Central and South America and Europe, whereas *B. bigemina* has been reported from Asia, Africa, Europe and the Far East (Bram, 2016). *B. divergens* is the least pathogenic species, but zoonotically important, and is present in Europe (Figueroa et al., 2010). The disease is characterized by extensive erythrocytic lysis resulting in anaemia, jaundice, pyrexia, haemoglobinuria, anorexia, abortion in

pregnant animals, neurological symptoms (rarely), and death in severe cases (Bram, 2016; Figueroa et al., 2010). The outcome of the disease depends on the virulence of the particular *Babesia* species and host factors, such as age, breed, and immune status. Bovine babesiosis can be treated with diminazine, or imidocarb, which are the only drugs effective against babesiosis available in the market (Figueroa et al., 2010).

2.12.3.2 Theileriosis

Theileriosis is an intracellular protozoan infection caused by members of the genus *Theileria.* These protozoa are transmitted by ixodid ticks and have a complex life cycle (Florin-Christensen, M. Schnittger, 2009). The geographical distribution of *Theileria* spp. is strictly dependent on the presence of tick vectors and is usually confined to tropical and subtropical countries. *Theileria* primarily infect domestic and wild ruminants. They produce significant diseases in cattle and small ruminants. Two important *Theileria* species, namely *T. annulata* (the causative agent of tropical theileriosis), and *T. parva* (the causative agent of East Coast Fever) are considered to be the most pathogenic species in cattle. Infections with other *Theileria* species, for example *T. mutans*, *T. taurotragi*, and *T. orientalis*, in bovines often remain without symptoms (Jongejan et al., 1986; Uilenberg, 1981). Many tick species, including *Hyalomma*, *Rhipicephalus* and *Amblyomma*, are involved in the transmission of theilerioses (Bishop et al., 2009). *Theileria* species invade WBC resulting in decreased immunity of the host. The development of *Theileria* species in tick vectors includes sexual reproduction which allows production of new variants, that enables them to escape the immune system of cattle (Katzer, 2010). Among *Theileria* species, *T. annulata* has numerous strains with a wide range of virulence, which are broadly distributed in different geographical regions of the world. *T. annulata* is mainly transmitted by members of the genus *Hyalomma* and produces a severe, potentially fatal disease in cattle, resulting in substantial economic losses in the dairy industry in Africa and Asia (Bishop et al., 2009). It has been observed that *T. annulata* produces severe illness in exotic and cross-bred cattle (*Bos taurus*), where the case-fatality rate can reach up to 80%, as compared to indigenous cows (e.g. *Bos indicus*), where the case-fatality rate is usually around 20% (Bishop et al., 2009; Ouhelli, 1991). For instance, the incidence of tropical theileriosis increased rapidly in India after the introduction of exotic cattle to increase milk production. Commonly, the disease occurs in its subclinical form, still resulting in considerable economic losses. Important clinical manifestations include increased body temperature, lymphadenitis, abnormal rapid respiration and heart rate, nasal discharge, anorexia, weight loss,

dullness and depression, severe pulmonary distress due to oedema, and death in severe cases. It can be treated with available drugs like buparvaquone, and parvaquone (Bishop et al., 2009).

2.13 Public health significance

There are many genera and species of ticks in the families Ixodidae (hard ticks) and Argasidae (soft ticks) that are of public health importance (CDC, 2013a). In humans, the diseases caused by viral and bacterial pathogens can vary from life threatening infections, such as Crimean Congo Hemorrhagic Fever (CCHF), tularemia and Rocky Mountain spotted fever (RMSF), to potentially chronic infections, like Lyme disease (de la Fuente et al., 2008). Ticks of the genus *Hyalomma* are considered as the principle vector for transmission of CCHF virus. *Hyalomma* ticks can also transmit *Rickettsia sibirica*, which causes Siberian tick typhus (CDC, 2013a). *Amblyomma* is well known for the transmission of tularemia, RMSF, ehrlichiosis, and boutonneuse fever. *Dermacentor* ticks can transmit tularemia, RMSF, Central European tick-borne encephalitis, Colorado tick fever and Siberian tick typhus. Ticks of the genus *Ixodes* can transmit Lyme disease, Russian spring-summer encephalitis and human granulocytic ehrlichiosis. *Rhipicephalus* ticks can transmit RMSF and boutonneuse fever (CDC, 2013a). Recently, new zoonotic viral diseases have emerged in Southeast Asia, such as Nipah virus infection (Chadha et al., 2006; Hsu et al., 2004).

Crimean Congo Hemorrhagic Fever (CCHF) virus can be maintained in all life stages of the tick vector by transstadial transmission and can be disseminated to the descendants by transovarial transmission. Wild and domestic animals, such as sheep, goat and cattle play a significant role in the natural cycle of the virus (WHO, 2016). The virus has been reported in mammals of Africa, Asia, and Europe where it produced mild fever (Sonenshine and Roe, 2014). Lyme disease is caused by *Borrelia burgdorferi* sensu lato, which is transmitted by tick of the genus *Ixodes* (*Ix. persulcatus* in China, *Ix. ricinus* in Europe and *Ix. scapularis* in North America). *Borrelia anserina* is transmitted by *Argas persicus* to poultry, causing avian borreliosis in a wide spread of tropical and subtropical countries (Hoogstraal, 1979). Infections with *Babesia* species are also gaining interest in human medicine due to their emerging zoonotic importance (Savic et al., 2014).

2.14 Economic significance of ticks and TBDs

Ticks cause substantial economic losses in the livestock sector in a number of different ways including direct and indirect production losses (which have been explained in detail earlier), costs incurred in controlling ticks and TBDs, in conducting research and training and providing

extension work to overcome these issues. The economic losses caused by ticks and TBDs vary widely with respect to spatiotemporal factors due to differences in husbandry practices and production systems, breed types, disease control policies and programmes (Biswas, 2003; Jonsson et al., 2008; Mukhebi, 1996). The economic loss caused by ticks and TBDs is estimated to be billions of US-Dollars (USD) annually (Jongejan and Uilenberg, 2004). Numerous studies have been conducted to estimate economic losses incurred by livestock sector due to ticks and TBDs. For instance, a FAO report (de Castro, 1997) estimated the annual global production losses rendered by ticks and TBDs at about 14–19 billion USD. Griffiths and McCosker (1990) reported ticks as the most important pests in the livestock industry globally, causing losses estimated at 7 billion USD per annum. Recent studies in Australia (Sackett and Holmes, 2006), and India (Minjauw and McLeod, 2003) have also assessed production losses at 26 million USD and 499 million USD per year, respectively. More importantly, increased susceptibility of exotic (improved) cattle breeds to tick infestation and TBDs somehow restricted their introduction in most parts of the country. In humans, vector-borne diseases caused over 148,000 deaths and more than 12.5 million disability-adjusted life years worldwide in 2002 (Beaglehole et al., 2004).

2.15 Current status of TBDs in Pakistan

In Pakistan, babesiosis (caused by *B. bovis*, and *B. bigemina*), theileriosis (*T. annulata* in buffalo and cattle, *T. ovis* and *T. lestoquardi* in sheep and goat), and anaplasmosis (*A. marginale* and *A. centrale*) are the most important TBDs of domestic animals (Ashraf et al., 2013; F. Iqbal et al., 2013; Jabbar et al., 2015; Khattak et al., 2012; Saeed et al., 2015). These tick-borne pathogens are possibly transmitted by hard ticks of the genera *Hyalomma*, *Rhipicephalus*, *Dermacentor*, and *Haemaphysalis* (Ali et al., 2013; Durrani and Kamal, 2008; Ghosh et al., 2007; Jabbar et al., 2015; Tasawar et al., 2014). The current status of the distribution of important TBDs along with their causative agents in large and small ruminants in Pakistan is given in Tables 2.3 and 2.4, respectively. So far, only one study has exposed the role of *Hyalomma* spp. in the transmission of *T. annulata* using molecular technique (Ali et al., 2013), while the role of other tick species in the transmission of tick-borne pathogens in Pakistan still needs to be investigated.

Table 2.3 Studies conducted to estimate the prevalence of various TBDs in bovines in different districts of Pakistan

Study area	Tick-borne pathogen	Study duration	Detection method	Prevalence		Reference
				Cattle	Buffaloes	
Attock, Islamabad	*A. marginale*	Sep 1999 to May 2001	BS	17.3 (53/307)	12.9 (20/155)	(Khan et al., 2004)
	Babesia spp.			0.65 (2/307)	0 (0/155)	
	T. annualata			0.98 (3/307)	0.6 (1/155)	
Bahawalnagar, Burewala, Kohat, Layyah, Multan, Peshawar	*Anaplasma* spp.	May to Sep 2011	PCR-RFLP	NA	41 (116/281)	(Ashraf et al., 2013)
	A. marginale				17 (20/116)	
Bahawalnagar, Bhakar, Layyah, Multan, Muzaffar Garh, Vehari	*B. bovis*	Jan to Aug 2010	BS	2.7 (4/144)	NP	(Zulfiqar et al., 2012)
			PCR	17.1 (18/105)	23.1 (9/39)	
	T. annualata	Information missing	BS PCR	3 (4/144) 19 (28/144)	NP	(Shahnawaz et al., 2011)
Charsadda, Swabi	*B. bigemina*	Jan 2010 to Dec 2011	BS	19 (19/100)	NA	(Ahmad et al., 2014)
	B. bovis			11 (11/100)		
Faisalabad	*Theileria* spp.	May-Jun 1982	BS	100 (3/3)	NA	(Ashfaque et al., 1983)
Faisalabad	*T. annualata*	Mar 1993 to Sep 1998	CS & BS	79.5 (89/112)	NA	(Muhammad et al., 1999)
Faisalabad, Jhang, Khanewal	*T. annualata*	Jul, Aug 2007	PCR	*Ha*: 50 (10/20) *Hd*: 20 (4/20)	NA	(Ali et al., 2013)
Hyderabad	*A. centrale*	Oct 1990 to Dec 1991	BS	7 (7/100)	11/100	(Buriro et al., 1994)
	A. marginale			11 (11/100)	19/100	
	Babesia spp.			1 (1/100)	1 (1/100)	
	Theileria spp.			3 (3/100)	5 (5/100)	
Hyderabad	*A. centrale*	Feb to Apr 2004	BS	9.2 (23/250)	8.4 (21/250)	(Rajput et al., 2005)
	A. marginale			22 (55/250)	13.6 (34/250)	
Karachi	*Babesia* spp.	Sep 1984 to Feb 1985	BS	4.2 (4/95)	1.4 (3/219)	(Haider and Bilqees, 1987)

Karachi	*A. marginale*	Nov 1984 to Dec 1985	BS	60 (30/50)	60 (60/100)	(Haider and Bilqees, 1988)
Karachi	*A. marginale*	Apr to Oct 2011	BS	NA	9 (9/100)	(Bhutto et al., 2012)
	B. bovis				3 (3/100)	
	T. annualata				2 (2/100)	
Kasur	*Babesia* spp.	Jul 2003 to Jun 2004	BS	2.5 (5/200)	NA	(Zahid et al., 2005)
	T. annualata			24 (48/200) 15 (30/200)		
Kasur	*B. bigemina*	Information missing	BS PCR	6 (6/100) 13 (13/100)	NA	(Durrani and Kamal, 2008)
	B. bovis		BS PCR	3 (3/100) 7 (7/100)		
	T. annualata		BS PCR	14 (14/100) 36 (36/100)		
Khanewal	*Anaplasma* spp.	May 2011 to April 2012	BS	4.1 (34/836)	4.29 (30/700)	(Sajid et al., 2014)
Khushab, Rawalpindi, Sargodha	*A. marginale*	Sep 2009 to Aug 2010	BS	5.8 (61/1050)	NA	(Atif et al., 2012)
			cELISA	31 (326/1050)		(Atif et al., 2013)
	B. bigemina	Sep 2009 to Aug 2010	BS	4.8 (50/1050)	NA	(Atif et al., 2012)
	T. annualata			5.14 (54/1050)		
Kohat, Peshawar	*T. annualata*	Nov 2010 to Feb 2011	BS PCR	5.3 (5/95) 33.7 (32/95)	NA	(Khattak et al., 2012)
KPK (different areas)	*A. marginale*	Jun to Jul 2003	BS	15.1 (8/53)	26.1 (17/65)	(Talat et al., 2005)
Lahore	*Theileria* spp.	Jul to Sep 2003	BS	NA	17 (107/600)	(Durrani et al., 2006)
Malakand Agency	*B. bigemina*	Information missing	BS	5.2 (42/794)	NA	(Ahmad et al., 2006)
Malakand Agency	*Babesia* spp.	Information missing	BS	6.6 (73/1100)	NA	(Ahmad and Hashmi, 2007)
Okara, Sheikhupura	*T. annualata*	Information missing	PCR	66.1 (41/62)	50 (20/40)	(M. K. Khan et al., 2013)
Peshawar	*A. centrale*	2001	BS	3.86 (11/285)	NA	(Afridi et al., 2005)
	A. marginale			4.2 (12/285)		
	B. bigemina			1.75 (5/285)		

	B. bovis			2.80 (8/285)		
	T. annualata			1.4 (4/285)		
Sahiwal	*Babesia* spp.	May to Jul 2005	BS	7.2 (30/415)	NA	(Niazi et al., 2008)
Sahiwal	*B. bigemina* & *B. bovis*	Jun to Aug 2005	BS PCR	18 (18/100) 18 (18/100)	NA	(Chaudhry et al., 2010)
	B. bovis		PCR	11 (11/100)		
Sahiwal	*T. annualata*	Apr to Sep 2009	BS	38.3 (115/300)	NA	(Qayyum et al., 2010)
Sargodha	*A. marginale*	Aug 2008 to Jul 2009	BS	9.7 (34/350)	NA	(Atif et al., 2012)
	B. bigemina			6.57 (23/350)		
	T. annualata			6.7 (24/350)		

BS = Blood smear; CS = Clinical signs; PCR = Polymerase chain reaction; *Ha* = *Hyalomma anatolicum*; *Hd* = *Hyalomma dromedarii*; NA = Not applicable; NP = Not provided

Table 2.4 Studies conducted to estimate the prevalence of various TBDs in small ruminants in different districts of Pakistan

Study area	Tick-borne pathogen	Study duration	Detection method	Prevalence		Reference
				Sheep	Goat	
Attock, Islamabad	*Theileria* spp.	Nov 2008 to Jul 2009	BS	7.36 (7/95)	3.8 (7/184)	(Irshad et al., 2010)
KPK (different areas)	*A. ovis*	Jun-Jul 2003	BS	13.2 (19/136)	9.59 (7/73)	(Talat et al., 2005)
Okara	*T. annulata*	Information missing	BS	16.5 (66/400)	NA	(Zia-ur-Rehman et al., 2010)
Bahawalnagar, Khanewal, Layyah, Multan, Muzaffar Garh, DGK, Vehari	*Babesia* spp.	Information missing	PCR	50 (20/40)	24 (16/67)	(Iqbal et al., 2011)
Lahore	*Theileria* spp. *T. ovis* *T. lestoquardi*	Spring & summer 2007	BS PCR	22 (44/200) 35 (70/200) 27.5 7.5	NA	(Durrani et al., 2011)
	T. ovis *T. lestoquardi*		PCR	*Rh*: 65.8 (27/41) *Hy*: 66.6 (30/45)		
Lahore	*Theileria* spp.	Nov 2005 to Oct 2006	BS	8.2 (21/256)	13.9 (38/273)	(Naz et al., 2012)
DGK, Layyah, Multan, RYK, Kohat	*T. ovis*	Information missing	BS PCR	1 (2/99) 11 (11/99)	0 1 (1/111)	(Durrani et al., 2012)
Okara	*B. ovis* *T. ovis*	May to July 2011	BS PCR BS PCR	29 (58/200) 55 (110/200) 37 (74/200) 58 (115/200)	NA	(Shahzad et al., 2013)
Multan, Kohat	*T. lestoquardi* *T. ovis*	Information missing	RLB	11.7* (21/196) 9.7 *(19/196)		(F. Iqbal et al., 2013)
Kohat, Peshawar	*T. lestoquardi*	Information missing	PCR	4.5 (2/44)	2.5 (3/121)	(Saeed et al., 2015)

DGK = Dera Ghazi Khan; RYK = Rahim Yar Khan; KPK = Khyber Pakhtunkhwa; BS = Blood smear; CS = Clinical signs; PCR = Polymerase chain reaction; NA = Not applicable; *Hy* = *Hyalomma*; *Rh* = *Rhipicephalus*; RLB = Reverse line blot; * = The authors did not mention the prevalence of *Theileria* spp. in sheep and goat separately

2.16 Diagnostic methods for TBDs

Ticks can harbor more than one pathogen, which can make the diagnosis of TBDs difficult. For a definitive diagnosis of a TBD, laboratory confirmation is required (FAO, 2016). Currently, a number of different approaches, ranging from very simple techniques like blood smear examination to highly developed advanced methods, such as PCR, are being used for the diagnosis of TBDs. Clinical signs and blood smear examination by microscopy (e.g., presence of piroplasms of *Babesia*, and *Theileria* spp. in RBCs, or schizonts of *Theileria* spp. in leucocytes, and inclusion bodies of *A. centrale* in the center and *A. marginale* on the margins of RBCs) have been the most commonly practiced diagnostic procedures. Besides these methods, numerous serological assays e.g., an enzyme-linked immunosorbent assay (ELISA), and an indirect immunofluorescence antibody technique (IFAT) for *Babesia* spp. (Figueroa et al., 2010) as well as *Theileria* spp. (Bakheit et al., 2004; Darghouth et al., 2004) and a competitive ELISA using the MSP-5 antigen (Torioni De Echaide et al., 1998) and IFAT (Gale et al., 1996) for *Anaplasma* spp., have been applied in epidemiological surveys of various TBDs. Although a number of different PCR methods have been established for the diagnosis of anaplasmosis, babesiosis (Carelli et al., 2007; Gale et al., 1996; Kim et al., 2007; Torioni De Echaide et al., 1998) and theileriosis (Gubbels et al., 1999)(Gomes and Inacio, 2015), these approaches are not commonly practiced in developing countries like Pakistan (Jabbar et al., 2015). Possible reasons may include the higher costs of reagents, and/or lack of expertise/knowledge needed to apply these advanced diagnostic methods (Jabbar et al., 2015).

The majority of the researchers in Pakistan relied on blood smear examination for the diagnosis of TBDs in their studies and PCR has only been applied in a small number of studies for the detection of *Babesia* spp. (Chaudhry et al., 2010; Durrani and Kamal, 2008; Zulfiqar et al., 2012), *T. annulata* (Durrani et al., 2008; M. K. Khan et al., 2013; Khattak et al., 2012; Shahnawaz et al., 2011) and *Anaplasma* spp. (Ashraf et al., 2013). Furthermore, only one study used a serological test (cELISA using the MSP-5) to estimate the seroprevalence of anaplasmosis in cattle in Pakistan (Ali et al., 2013). Besides the use of traditional and advanced diagnostic tools, many researchers studied the haematological and biochemical profile of clinically affected buffaloes and cattle (Ahmad and Hashmi, 2007; Atif et al., 2012; Khattak et al., 2012; Qayyum et al., 2010; Shahnawaz et al., 2011).

2.17 Tick control methods

Tick control is essential to protect animals from distress and production losses, secondary infections at the site of lesions, toxemia, paralysis, impairment to hides, and of utmost importance, infection with tick-borne pathogens. It also helps to minimize the spread of tick species and TBDs to unaffected regions or continents.

2.17.1 Use of acaricides

A number of different acaricides have been used for tick control worldwide. Acaricides are synthetic chemical pesticides used, particularly, against ticks and can be applied in different ways such as dipping, spraying on animals and their surroundings, pouring on animals and incorporation in polyvinylchoride plastic ear tags (George et al., 2008; Willadsen, 2006). The majority of the acaricides belongs to organophosphates (e.g. chlorfenvinphos), formamidines (e.g. amitraz), synthetic pyrethroids (e.g. flumenthrin), phenylpyrazoles (e.g. fipronil), and benzylphenyl ureas (e.g. fluazuron). Although acaricides can be very effective if applied properly and in right concentrations, there are some disadvantages, which include the risk of acute poisoning of animals and animal handlers, the presence of residues in animal products such as meat and milk, environmental pollution, especially of water sources, resistance in ticks, especially when acaricides are applied in low concentrations, and last but not the least the cost of application. A major problem of the selection of acaricide resistant ticks has been reported in tick control efforts worldwide (Abbas et al., 2014; Mendes et al., 2007). Many predictive models have been developed in advanced countries, which forecast the best times for acaricidal treatment on the basis seasonal activity and population dynamics of tick species (Mount, 1991; Randolph and Rodgers, 1997; Schmidtmann, 1994). In developing countries like Pakistan, many farmers use local remedies including feeding of grinded Taramira (*Eruca sativa*) to the tick infested animal or topical application of common salt (Muhammad et al., 2008).

2.17.2 Biological and cultural control

There are various biological control methods, which have been practiced in the past and can be applied equally against parasitic as well as free-living stages of ticks. Removal of a particular type of vegetation has been practiced in South Africa and the USA to control *Ix. rubicundus* and *Am. americanum*, respectively. Similarly, rotation of pastures has been exercised to control *Rh. microplus* in Australia, but the same could be used for other one-host tick species. Another approach could be the removal of hosts of a particular tick stage. Although the implementation of

this method is quite difficult, it has been suggested to control three-host ticks such as *Hylomma* spp. in Asia and Europe, and *Am. hebraeum*, *Ix. rubicundus* and *Rh. appendiculatus* in Africa.

Development of tick resistant breeds of cattle, e.g. Zebu and Sanga cattle, the local breeds of Asia and Africa, has been proved a successful approach in controlling ticks (Levin, 2015b). The Sahiwal breed of cattle in Pakistan is well known in the world for its strong tick resistant ability (Muhammad et al., 2008; Sajid et al., 2009). The control of *Rh. microplus* in Australia has been revolutionized after the introduction of Zebu cattle (Levin, 2015b).

Additionally, ticks have many natural enemies including birds, shrews, rodents, ants etc., which play their role in decreasing the number of parasitic as well as free-living ticks. For example, oxpeckers (*Buphagus* spp.) feed on attached ticks and decrease the tick burden on animals (Levin, 2015b; Muhammad et al., 2008). Integrated livestock farming, e.g. poultry and dairy husbandry, can also contribute to biological tick control (Muhammad et al., 2008). As a result of the development of acaricidal resistance and increasing public health concerns with reference to residues in meat and milk, biological control is gaining popularity in future integrated tick control programs (Dennis and Piesman, 2005; Samish et al., 2004).

2.17.3 Tick vaccines

Traditional control strategies against ticks and tick-borne pathogens, such as the use of harmful acaricides and the destruction of wildlife reservoirs, are becoming increasingly objectionable (Piesman and Eisen, 2008). Vaccines can provide the most proficient and cost effective means of prevention and control, especially when the risk of infection is high and the outcome is severe (Monath, 2013). The conceptual framework of using tick vaccines is to control the tick species locally, and provide a safe and environmental friendly substitute for acaricides (de la Fuente et al., 2007; Torina et al., 2014). Another reason for using vaccines as tick control measure is that they do not only impair tick feeding, but also stops the transmission of tick-borne pathogens to the host (de La Fuente et al., 2015; Hovius et al., 2007; Labuda et al., 2006). Therefore, development of a single tick vaccine might be a much better choice than developing many vaccines against all tick-borne pathogens, which are transmitted by that tick species (Sprong et al., 2014). Like other anti-parasitic vaccines, the identification of potential antigens, however, remained a stumbling block in the production of efficacious anti-tick vaccines (Hope et al., 2010; Willadsen, 2008). Anti-tick vaccines targeting *Rh. microplus*, namely TickGARD Plus and Gavac Plus, have been effectively

launched in the dairy industry in Australia. These vaccines utilized Bm86 antigen, which is a glycoprotein molecule from outer membrane of gut digest cells of the feeding ticks and has proved to be an efficacious antigen eliciting an immune response that protects against ticks, and were further developed using recombinant DNA techniques (Willadsen, 2008). Later on, a similar vaccine was produced in Cuba (Rodríguez et al., 1995). In Pakistan, a desirable immunogenicity of a vaccine targeting *Rh. microplus* has been achieved by in vitro studies (Akhtar et al., 1999; Akhtar and Hayat, 2001). In the past, the use of anti-tick vaccines has significantly decreased the incidence of babesiosis in bovines (Valle et al., 2004).

2.18 Control strategies against tick-borne pathogens

Presently, numerous control strategies, which include tick control, drug treatment and vaccination, are globally used to minimize the economic losses caused by TBDs (Muhammad et al., 2008).

2.18.1 Chemotherapy against tick-borne pathogens

Tick-borne pathogen infections are generally treated with either antibiotics or antiprotozoal drugs depending on the type of pathogen involved. Commonly used antibiotics against tick transmitted bacterial pathogens, particularly *Anaplasma* spp., include tetracycline and doxycycline. Clinical cases of babesiosis are treated with imidocarb dipropionate and/or diminazine aceturate. Infections with *Theileria* spp. are commonly treated with parvaquone and/or halofuginone. Antiprotozoal drugs are also used for prophylaxis. Use of medicines to control TBDs is a costly choice and it does not always completely clear infections, which may lead to the development of resistance by the pathogens and leave the animal in a carrier state. (Stone, 1989)(Taylor, 2007).

2.18.2 Vaccination against tick-borne pathogens

Due to inadequacies in prevention and control measures against TBDs based on chemotherapy and tick control, the requirement of potentially effective vaccines against TBDs is rapidly increasing. Live vaccines against tick-borne pathogens have been made available for last two decades, but in spite of their clear efficacy, they have not been adopted worldwide. Lack of facilities and resources for vaccine production and supply on large scale, as well as fear of the introduction of new pathogen strains into regional tick populations have generally restricted the utilization of these vaccines (Morisson and Mc Keever, 2006).

To minimize the incidence of theileriosis, a combination of planned tick control measures and immunization against *Theileria* spp. is required. However, these strategies are yet to be effectively

employed on a broad level in endemic zones (OIE, 2014a). High quality vaccines for *T. annulata* and *T. parva* have already been produced. For *T. annulata*, live attenuated vaccine is produced using schizont-infected cell lines from cattle during in-vitro culture. For *T. parva*, the required level of immunity is achieved by injecting a subcutaneous dose of tick-derived sporozoites in combination with any long-acting tetracycline. Treated animals gain a lifetime immunity against *T. parva* infections (OIE, 2014a). Recently, studies have aimed at the development of subunit vaccines for *Theileria* species (Morisson and Mc Keever, 2006). For *Babesia* spp., live attenuated vaccines against *B. bovis*, *B. bigemina* or *B. divergens* are produced in many countries using blood of the infected animals or from in-vitro culture, however, there are issues regarding the safety of these vaccines (OIE, 2010).

Although anaplasmosis, caused by *A. marginale*, has a worldwide distribution, there is no conventional vaccine available. Blood-based live vaccines have been used in tropical regions to protect cattle, but, like vaccines against *Babesia* spp., there are always certain chances of transmission of other pathogens to the vaccinated animal (Kocan et al., 2003). A live vaccine against *A. centrale* is the most widely used vaccine, which also provides partial immunity against *A. marginale*, however, this vaccine is also not safe. The use of this vaccine for the control of *A. marginale* is only recommended in those countries where *A. centrale* is endemic (OIE, 2015). According to the recommendations of World Organisation for Animal Health (OIE), the use of the vaccines against *Anaplasma* spp. and *Babesia* spp. mentioned above should be restricted to calves, as innate immunity will decrease the risk of some vaccine reactions that may need therapy with tetracycline or imidocarb. A vaccine against anaplasmosis is used in many states of the USA since May 2000, but still needs to be registered by the United States Department of Agriculture (USDA). This Experimental Anaplasmosis Vaccine is the only killed vaccine against *A. marginale* and was developed at Louisiana State University, Agriculture Center's Veterinary Science Department, during late nineties.

2.19 Prevention and control methods against ticks and TBDs in Pakistan

In developing countries, like Pakistan, grooming is the most commonly used tick control strategy at traditional rural dairy farms (Masika et al., 1997; Mondal et al., 2013). It involves removal of ticks one by one manually and to throw them into the fire made with dried animal dung. Many farmers used trichlorfon and ivermectin during high-risk months (June to September) of the year to control external parasites, particularly ticks. Semi-commercial and commercial dairy farms use

cypermethrine solutions for spraying the animals and their surroundings. So far, tick control has been considered the most widely and frequently used method of controlling TBDs in animals, but the reliance on acaricides is declining due to the rapid development of acaricide resistance in various tick species (Abbas et al., 2014) and public health concerns regarding acaricidal residues in milk and meat (Kay and Kemp, 1994; Samish et al., 2004). In recent years, a number of therapeutic or prophylactic trials have been conducted to measure the efficacy of diminazene in combination with imidocarb, buparvaquone and oxytetracycline against babesiosis, theileriosis and anaplasmosis, respectively (Atif et al., 2012; Muhammad et al., 1999; Niazi et al., 2008; Qayyum et al., 2010; Zahid et al., 2005). Due to lack of information on tick-borne pathogens species involved in the occurrence of TBDs, a fixed program for prophylaxis is not practiced in the country.

3 Materials and methods

3.1 Study site

The whole country was divided into five agro-ecological zones on the basis of aridity data set retrieved from CGIAR-CSI Global-Aridity and Global-PET Database (Zomer et al., 2008, 2007) . Of the five agro-ecological zones, two major zones (the semi-arid and the arid zone) that cover more than 80% area of the country, including Punjab province, were selected for sampling. The climate time-series data of the semi-arid and the arid zone is given in Table 3.1. The study focused on Punjab province (Figure 3.1), which is the most populous province in terms of human and animal population. It is located between 28° to 33° N and 70° to 74° E. The name "Punjab" is derived from two Persian words, which mean the Land of Five Rivers. These five rivers are the tributaries of the Indus River namely Beas, Chenab, Jhelum, Ravi and Sutlej. Punjab province is quite rich in agricultural production because it has one of the largest irrigation systems of the world with approximately 3,000 irrigation channels. Although it lies mainly on plains, it also contains hilly areas in the extreme Southwest and in the Northwest. Some parts of the Cholistan desert are also located in the territory of the province. All four seasons occur in the province, but during late summer, the weather becomes humid, which is very suitable for tick multiplication and infestation. Although, weather extremes are noticeable from the hot and barren South to the cool hills of the North, but generally the temperature ranges between −2°C and 45°C and can reach up to 50°C in summer and −5°C in winter. The province is the biggest contributor to the national GDP with 59% in 2014 (Ministry of Finance, 2015). It shares almost half of the total livestock population in Pakistan, i.e. 18.8 million heads of cattle, 22.0 million buffaloes, 24.0 million goats and 7 million sheep[4].

[4] Source: Ministry of National Food Security & Research. Estimated figures based on inter census growth rate of Livestock Census 1996 & 2006
http://www.livestockpunjab.gov.pk/View.aspx?Type=TopMenu&itemId=1737. Accessed on August 1, 2015.

Table 3.1 Weather data recorded at the stations of Pakistan Meteorological Department

Months	Semi-arid zone (altitude: 188-404 m)				Arid zone (altitude: 67-180 m)			
	Mean daily temperature (°C)		Rainfall (mm)	Relative humidity (%)	Mean daily temperature (°C)		Rainfall (mm)	Relative humidity (%)
	Max±SD	Min±SD		Mean±SD	Max±SD	Min±SD		Mean±SD
Aug 2013	30.5±3	22.5±1.8	340	79.0±13.1	37.2±2.5	27.5±1.4	51	68.6±10.7
Sep 2013	32.2±1.5	21.6±1.7	27.2	70.5±11.4	37.6±1.6	25.6±1.1	0	60.9±6.1
Oct 2013	29.6±1.9	17.9±3.1	19.1	68.2±12.3	35.4±2.3	21.9±3.5	1	60.7±7.2
Nov 2013	23.8±1.7	9.6±2	6.5	67.9±10.4	28.3±2.3	12.8±1.5	1	64.1±8.1
Dec 2013	18.6±4	4.8±2.2	5.4	71.4±14.0	22.9±4.6	8.3±3	0	67±11.4
Average	26.9±2.4	15.3±2.2	79.6	71.4±12.2	32.3±2.7	19.2±2.1	10.6	64.3±8.7

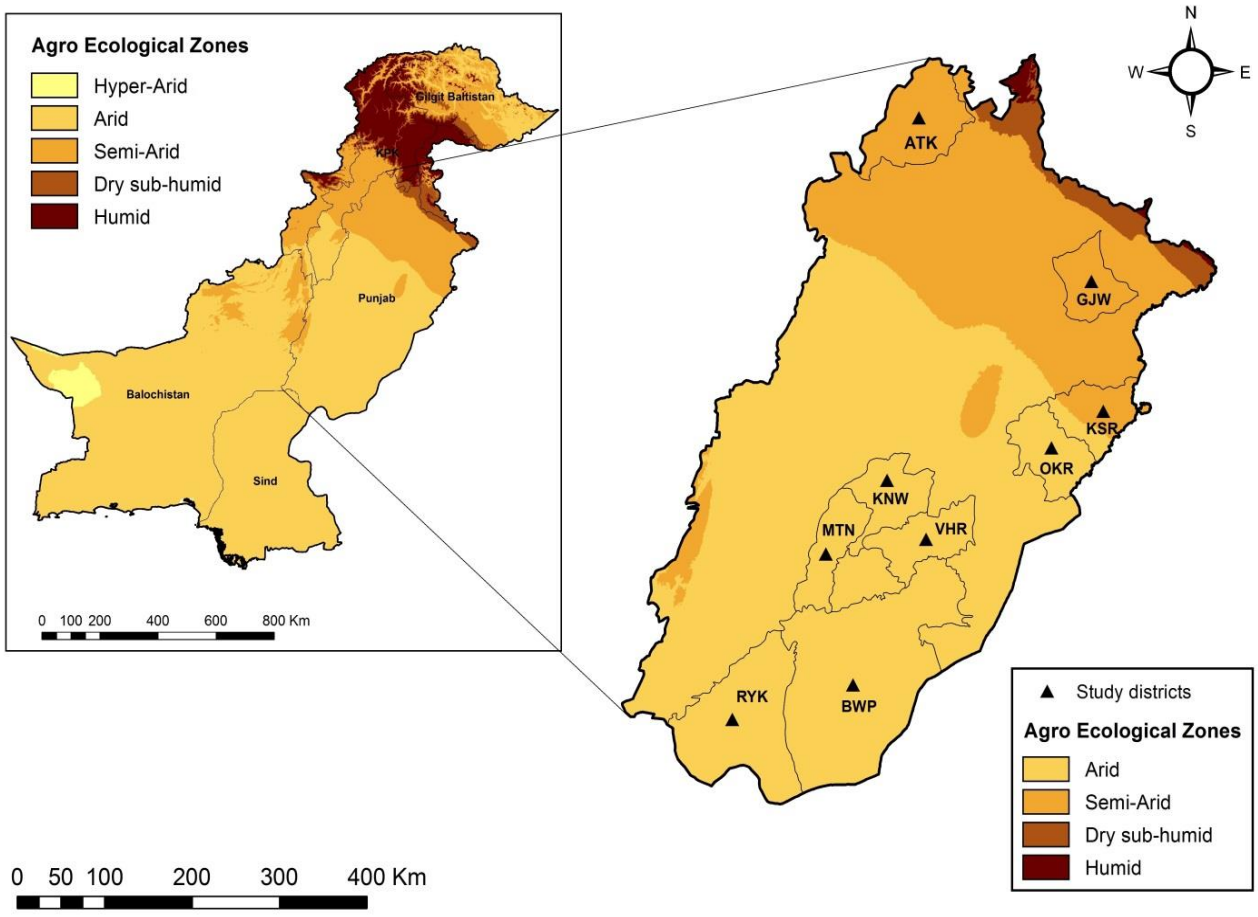


Figure 3.1 Map of Punjab province within Pakistan and the districts from where tick samples were collected

3.2 Study design

A descriptive cross-sectional study was designed to investigate the distribution of ticks infesting ruminants on livestock farms. The sample size was calculated for large populations assuming 50% prevalence at the 95% confidence level and 10% desired precision, which estimated that at least 97 livestock farms should be included (Cannon and Roe, 1982). The number was adjusted up to 108 livestock farms according to the administrative units and a multistage sampling technique was used to select the farms: In the first stage, nine (25%) out of 36 districts were selected. Then from each district six Union Councils were chosen and from each Union Council two villages were selected. In each selected village, one livestock farm was visited for tick sampling and data collection.

The livestock farms were selected in 9 districts, namely Attock (ATK), Gujranwala (GJW), Kasur (KSR), Okara (OKR), Khanewal (KNW), Multan (MTN), Vehari (VHR), Bahawalpur (BWP) and Rahim Yar Khan (RYK) (see Figure 3.1) from two different agro-ecological zones, namely the semi-arid and the arid zone. The former three districts are located in the semi-arid zone and the others are in the arid zone.

All the districts are important livestock husbandry regions. Two of them (BWP and RYK) have the highest small ruminant population (1.8 million and 1.5 million, respectively) in Punjab and one (KSR) is well known for its buffalo population, which is more than 1 million. Three of them (KSR, BWP and OKR) are adjacent to India and two (ATK and RYK) are important livestock trade zones, which connect the northern part of the country with the southern part. Bahawalpur district is the largest district of Punjab province with a total area of 24,830 km^2, of which 66% is covered by the Cholistan desert, which is connected to the Thar desert of India.

3.3 Collection and preservation of ticks

A brief introduction on the study was given before obtaining the consent of the owner of the farm on his participation in the study. All tick specimens were collected during 2013 except for the Multan district, from which the samples were recollected in June of the following year, due to unforeseen reasons[5]. Ticks were collected from buffaloes, cattle, goats and sheep. To estimate the herd prevalence, two animals from each ruminant species varying in age and sex present at the

[5] Tick samples from Multan region, except for one farm, were re-collected in the month of June of the following year because some of the samples were lost in a major road accident while the researcher was travelling to send the shipment from Lahore to Berlin through World Courier®.

farm were randomly selected based on the assumption, that if ticks would be present on a farm, then at least 50% of the animals on that farm would be infested (Lorenz, 1990). Thus, a minimum of two and a maximum of eight animals were investigated on each farm.

The following predilection sites were examined for the presence of ticks in each animal: 1) ears 2) brisket (dewlap in case of cattle) 3) withers 4) knees 5) udder in case of females and testes in males along with perineum region and 6) tail (Londt et al., 1979). The total tick burden on one side of the animal except for the tail region was estimated according to patch sampling, which has been suggested by Mooring & McKenzie (1995) for obtaining measures of relative tick burden rather than absolute tick counts. This method is comparatively quick and easy to perform in a field situation. The basis for patch sampling is the well-known fact that ticks tend to concentrate on certain predilection sites on the body surface of the host (Baker and Ducasse, 1967; Howell et al., 1978; Kaiser et al., 1982; Sachs and Debbie, 1969). No attempt was made to identify species, sex or stage of the ticks during tick burden estimation.

A representative proportion of all the different types of ticks were collected from predilection sites using a simple tick remover (Ticked Off™, New Hampshire, USA) according to the manufacturer's instructions. A blunt steel forceps was also used for large sized ticks (CDC, 2013). Ticks were transferred to Safe-Lock Eppendorf® tubes added with 70% ethanol, labelled with a unique sample ID, which was comprised of the farm ID, the host species and the body location. A separate tube was used to collect ticks from each predilection site and all the different types of ticks present there were collected into the respective tube. The information regarding each specimen and host related factors including species, breed, gender and age were recorded on a predesigned form (Appendix A). The age of the animal was recorded on the basis of information provided by the owners.

3.4 Investigation of risk factors

To investigate determinants associated with tick prevalence, a questionnaire was developed, which contained 21 closed and 10 open-ended questions. The questionnaire was divided into three parts: (A) farm-related information, (B) tick-related information and (C) herd management-related information. The variables considered were: (i) farm type, (ii) number of persons working on farm, (iii) animal species present on farm, (iv) purpose of farming, (v) educational status of the owner, (vi) months of tick infestation, (vii) months of highest infestation, (viii) use of drugs for tick control, (ix) name of acarcide, animal species treated, application method and months of

application, (x) use of acaricides according to age group, (xi) reasons for not using acaricides, (xii) other strategies for ticks control, (xiii) change in the behavior of tick positive animal, (xiv) effect on milk production, (xv) frequency of veterinary or para-veterinary staff, (xvi) housing facilities, (xvii) housing materials, (xviii) floor type, (xix) presence of boundary wall, (xx) tethering practice, (xxi) feeding method, (xxii) feed storage, (xxiii) distance to the nearest livestock farm, (xxiv) frequency of disposal of animal dung, (xxv) presence of vegetation on farm, (xxvi) quarantine measures and (xxvii) history of mortality. The questionnaire was developed in English language, but administered in Urdu and Punjabi according to farmers' requirement to ensure that the farmer understood all the questions. The data were collected with the help of local veterinarians of the respective areas. The detailed questionnaire is presented in Appendix A. The tick samples were then shipped from Pakistan to Germany through World Courier® (Reference No. 650009635) for further testing.

3.5 Identification of ticks

Morphological identification of the ticks was subsequently performed using standard taxonomic keys and a multikey software, i.e. a computer-based polychotomous key (Estrada-Pena et al., 2004; Walker et al., 2005) at the Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin. The key uses a more versatile knowledge-based method of data representation and this allows the system to cope with incomplete or missing data. Additionally, original descriptions and re-descriptions of relevant tick species were also followed (Apanaskevich and Horak, 2005). The specimens were identified at the species level under a stereomicroscope with 40-fold magnification. The abbreviations for the identified tick genera were used as previously suggested by Dantas-Torres (2008).

3.5.1 PCR amplification of ITS2 and COX1 genes

To confirm the results of the morphological identification, fragments from the second internal transcribed spacer (ITS2; ~750 bp) gene from randomly selected specimens (n = 19) was amplified and sequenced as follows: ticks were washed with distilled water and subsequently homogenized using sterilized pestles in 1.5 ml Safe-Lock Eppendorf® tubes (Sigma-Aldrich Chemie Gmbh Munich, Germany) containing T1 buffer (180 µl), to which Proteinase K (25 µl) was added. DNA extraction was then performed using the NucleoSpin® Tissue Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendations for the purification of genomic DNA from insects. The purified DNA was quantified using the Take3 Micro-Volume

plate with Gen5 software using an Epoch Microplate Spectrophotometer (BioTek Instruments, Bad Friedrichshall, Germany). A partial fragment of the ITS2 gene was amplified using primers (ITS2-F) Metastriata IST-F (5'-AGGACACACTGAGCACTGATTC-3') and Metastriata IST-R (5'-ACTGCGAAGCACTTRGACCG-3').

The PCR was performed in a total reaction volume of 25 µl containing 2.5 µl of 10x Maxima Hot Start Taq Buffer, 2.5 µl of 1.5 mM MgCl2, 2.5 µl of 2mM dNTPs, 1 µl (10 µM) of each primer, 0.25 µl (2U/µl) of Maxima™ Hot Start Taq DNA Polymerase (Thermo Scientific™ (Karlsruhe) GmbH, Germany) and 2.5 µl of template DNA. The amplification was carried out in C1000™ Thermal Cycler (Bio-Rad Laboratories GmbH, München, Germany). The cycling conditions comprised a 5 min denaturation and polymerase activation step at 95°C, 40 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 50 s and a final extension step for 5 min at 72°C. A negative control was also used to authenticate the PCR reaction. The PCR amplicons were loaded into 1.5% agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) with GRGreen Nucleic Acid Stain (LABGENE Scientific, Châtel-St-Denis, Switzerland). To visualize the PCR amplicons in UV transilluminator, GeneSnap from SynGene version 7.12 was used. The GeneRuler 100 bp DNA Ladder (Thermo Scientific™ (Karlsruhe) GmbH, Germany) was used to compare the sizes of the amplicons.

The PCR products were purified using the Zymoclean™ Gel DNA Recovery Kit with capped columns (Zymo Research Corporation, Freiburg, Germany) according to the manufacturer's instructions. The quantity and quality of the purified DNA samples (< 700 bp) were measured with an Epoch Microplate Spectrophotometer (BioTek®, Bad Friedrichshall, Germany). The 260/280 ratio was 1.93 ± 0.19 (Mean±SD), indicating excellent purity. The concentration of the DNA was verified using agarose gel electrophoresis. The purified samples were then submitted to LGC Genomics GmbH, Berlin, Germany for sequencing with the forward primer as used in PCR amplification. The resulting sequences were then confirmed phylogenetically through nucleotide BLAST search in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/blast/). Following confirmation of tick species identity by the ITS2 gene, a 1,592 bp fragment of the cox1 gene from five *Rh. microplus* specimens was amplified and sequenced using primers Rhcox1-F (5'-CCGCCTAAACTTCAGCCATT-3') and Rhcox1-R (5'-GTCTGAAAATGYTAATTGAGATCAAG-3') with identical PCR conditions, except the extension time, i.e. 100 s, as described for the amplification of the ITS2 gene.

3.6 Detection of Tick-borne Pathogens

3.6.1 Extraction of DNA from tick pools

After identification, the ticks were divided into 405 pools based on their species, locality of collection and the host, from which they were collected. Only ticks belonging to the same species and originating from the same animal were pooled together. Generally, three ticks from each pool were used for the extraction of DNA, but if the number was less, then all ticks were included. Fully engorged specimens were cut into two equal halves using a sterilized blade and only one half from each engorged tick was used for DNA extraction. Ticks were dried on filter paper and homogenized in 1.5 ml Safe-Lock Eppendorf® tubes in T1 buffer (180 µl) with Proteinase K (25 µl) using a sterilized pestle. The tubes were then placed at 56ºC for overnight incubation in a TMix shaking incubator (Analytik Jena AG, Jena, Germany). The RS-VA 10 vortexer with adjustable speed was used for proper mixing (Phoenix Instrument GmbH, Garbsen, Germany). DNA extraction was performed using a commercial kit (NucleoSpin® Tissue Kit, Macherey-Nagel GmbH & Co. KG, Düren, Germany) following to the manufacturer's protocol for the elution of high quality and quantity of DNA. The quality and quantity of the extracted DNA samples were measured in terms of 260/280 ratio using an Epoch Microplate Spectrophotometer (BioTek®, Bad Friedrichshall, Germany). All DNA extractions were stored at −20°C when not in use.

3.6.2 Amplification of DNA of tick-borne pathogens using PCR

Two sets of PCRs were performed on each DNA sample, one for the amplification of *Babesia* and *Theileria* DNA and the other for *Anaplasma, Ehrlichia* and *Rickettsia* spp. DNA. The PCR was performed in a total reaction volume of 25 µl, which consisted of 12.75 µl H_2O, 5 µl of 5X Phusion HF buffer with 1.5 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs, 1 µl of 10 pmol/µl of primers RLB-F2 and RLB-R2 (*Babesia* and *Theileria* amplification) or primers Ehr-F2 and Ehr-R2 (*Anaplasma*, *Ehrlichia* and *Rickettsia* amplification, Table 3.2), 0.25 µl (2 U/µl) of Phusion Hot Start II DNA Polymerase (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and 2.5 µl of DNA template.

For *Babesia* and *Theileria*, a touchdown PCR program was performed: two cycles of 98°C for 10 s (denaturation), 68°C for 20 s (annealing), and 72°C for 15 s (extension), followed by a succession of two-cycles with conditions identical to the previous cycles with the annealing temperature reduced by 2°C until it reached 58°C. A further 35 cycles were performed at 98°C for 10 s, 58°C

for 20 s, and 72°C for 15 s with a final extension step at 72°C for 8 min. For *Anaplasma*, *Ehrlichia* and *Rickettsia*, all the conditions were the same, except for the initial annealing temperature, which was 71°C then following the touchdown at 61°C. A positive and a negative control were also used to confirm the results. All amplifications were carried out in a C1000™ Thermal Cycler (Bio-Rad Laboratories GmbH, München, Germany). Amplicons were visualized on 1.5% agarose gels (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) stained with GRGreen Nucleic Acid Stain (LABGENE Scientific, Châtel-St-Denis, Switzerland) under UV light using GeneSnap from SynGene version 7.12. A GeneRuler 100 bp DNA Ladder (Thermo Scientific™ (Karlsruhe) GmbH, Germany) was used to compare the sizes of the amplicons.

Table 3.2 Features of primers (*Babesia*/*Theileria* and *Anaplasma*/*Ehrlichia*/*Rickettsia*) used in PCR reaction. The melting temperatures of primers were calculated using OligoAnalyzer 3.0

Primer*	Sequence	Melting temp (^{o}C)
RLB-F2	5'-GAC ACA GGG AGG TAG TGA CAA G-3'	57.9
RLB-R2	5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3'	53.7
Ehr-F2	5'-AGA GTT TGA TCC TGG CTC AG-3'	61.0
Ehr-R2	5'-Biotin-GAG TTT GCC GGG ACT TYT TCT-3'	69.5

Genus-specific primers, RLB-F2/RLB-R2, were used to amplify a fragment of 460–540 bp of the 18S SSU rRNA gene of the V4 region of *Babesia* and *Theileria* species (Gubbels et al., 1999). For identification of *Anaplasma*, *Ehrlichia* and *Rickettsia* species, the primers Ehr-F2/Ehr-R2 were used to amplify a fragment of approximately 500 bp of the 16S rRNA gene of the V1 region of *Anaplasma*, *Ehrlichia* and *Rickettsia* species.

3.6.3 Reverse line blot (RLB) assay

3.6.3.1 Preparation of the RLB membrane

Oligonucleotides of different species of *Babesia*, *Theileria*, *Anaplasma*, *Ehrlichia* and *Rickettsia* with N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl,*N*,*N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker (Isogen, Life Science, Maarssen, The Netherlands) were diluted in 150 µl 0.5 M $NaHCO_3$, pH 8.4. The oligonucleotide probes (n = 41) that were included in the RLB hybridization membrane to detect the TBPs are listed in Appendix B. A Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, MI, USA) was marked on 2 corners to facilitate its orientation. The membrane was activated by 10 min incubation in 10 ml freshly prepared 16 % (w/v) EDC-HCl (Carl Roth, Karlsruhe, Germany) at room temperature and subsequently rinsed

with demineralized water. The membrane was transferred to a clean MN45 miniblotter (Immunetics, Cambridge, Mass., USA) with a support cushion. The miniblotter was closed using the screws and placed in vertical position to remove the residual water from the slots by aspiration (vacuum). Each slot was then filled with 150 µl of the oligonucleotide probes, except for the first and the last slots which were loaded with drawing pen ink diluted 1:100 in 2 X SSPE. After incubation of 1 min at room temperature, the solutions were removed by aspiration in the same order as they were applied. The membrane was taken away from the blotter and was shifted to a washing tray to inactivate it with 100 ml 100 mM freshly made NaOH for 8 min. Afterwards the membrane was washed in 100 ml 2 X SSPE/0.1% Sodium dodecyl sulfate (SDS) at 60ºC for 5 min.

The membrane was then washed in 20 mM Ethylenediaminetetraacetic acid (EDTA), pH 8 for 15 minutes at room temperature under gentle shaking using a plastic container and stored in a sealed plastic bag added with 20 ml of 20 mM EDTA at 4ºC.

3.6.3.2 RLB assay

The membrane was incubated in a plastic container for 5 min in about 10 ml 2 X SSPE/0.1% SDS at room temperature. Ten µl of each PCR product originating from the same DNA pool were diluted in 140 µl 2 X SSPE/0.1% SDS and heated for 10 min at 100°C to denature the amplicons and then cooled on ice immediately for a minimum of 5 min. The samples were centrifuged at 13,000 rpm for 30 s. The membrane was placed over the miniblotter, with slots perpendicular to the line pattern of the applied probes so that the ink-lanes were located directly underneath the openings of the slots. A support cushion was placed under the membrane and the screws were tightened to avoid cross flow. The residual fluid was removed by aspiration and slots were filled with diluted PCR product avoiding the air bubbles. The first and last slots were filled with 2 X SSPE/0.1% SDS to prevent desiccation of the membrane. The hybridization was performed in HB-1000 Hybridizer Oven (Analytik Jena AG, Jena, Germany) for 75 min at 42ºC. After hybridization, samples were removed by aspiration and the membrane was removed from the blotter. The membrane was washed twice in pre-heated 2 X SSPE/0.5% SDS for 10 min at 50ºC in a water bath under gentle shaking and incubated with 10 ml 2 X SSPE/0.5% SDS + 2.5 µl streptavidin-POD (peroxidase labeled) (Boehringer, Mannheim, Germany) conjugate (1.25 U) for 30 min at 42ºC. A second washing step was repeated twice in pre-heated 2 X SSPE/0.5% SDS for 10 min at 42ºC in a water bath under gentle shaking. The membrane was washed twice with 2 X SSPE for 5

min at room temperature under gentle shaking. The membrane was then transferred to a plastic container and 10 ml ECL detection fluid (5 ml ECL1 + 5 ml ECL2) (GE Healthcare UK Limited., Amersham, Buckinghamshire, United Kingdom) were spread over the membrane, followed by a 1 min incubation under gentle shaking. The ChemoCam Imager 3.2 (INTAS Science Imaging Instruments GmbH, Göttingen, Germany) was used to detect chemiluminescent signals from the membrane. The sequential integrated mode was chosen for 20 scans (each after 1 minute - shutter time) using 1x1 binning. The image was exported as a TIF file to retain the high quality.

The miniblotter was cleaned with (neutral pH) detergent and rinsed with plenty of running water followed by distilled water and then left to dry. The support cushion was also cleaned with water.

3.6.3.3 Stripping of the RLB membrane

PCR products were removed from the membrane by two washes with about 100 ml pre-heated 1% SDS at 90°C for 30 min under gentle shaking. The membrane was thereafter washed once with approximately 100 ml 20 mM EDTA for 15 min at room temperature under gentle shaking and was stored at 4°C in a sealed plastic bag containing about10 ml 20 mM EDTA, pH 8 for further use.

3.6.4 Confirmation of the detected tick-borne pathogens

To confirm the results of RLB hybridization, selected samples representing each tick-borne pathogen species were selected and purified using the Zymoclean™ Gel DNA Recovery Kit with capped columns (Zymo Research Corporation, Freiburg, Germany) according to the manufacturer's instructions. The purified samples were then submitted to LGC Genomics GmbH, Berlin, Germany, for sequencing with the same forward primer as used in amplification of the tick-borne pathogen DNA. For further confirmation of *Rickettsia* spp., a larger fragment (~1175 bp) of the 16S ribosomal RNA gene was amplified with a different reverse primer, named Rickketsia-R2 (5'-CCT TCA GGT AAA ACC AAC TCC-3'). All the PCR conditions except the extension time, which was 35 s because of the larger product size, were similar as described previously in section 3.6.2. The amplified products were purified and sequenced from both sides using the same primers as used in the PCR. The sequencing results were finally confirmed phylogenetically through a BLAST search in the National Center for Biotechnology Information (NCBI) database.

3.7 Details of the aridity map dataset

According to the Consultative Group for International Agricultural Research (CGIAR), *"Aridity is usually expressed as a generalized function of precipitation, temperature, and/or potential evapo-transpiration (PET). An Aridity Index (UNEP, 1997) can be used to quantify precipitation availability over atmospheric water demand"*. Global mapping of the mean Aridity Index for the period of 1950-2000 at 30 arc sec (~ 1km at equator) spatial resolution was calculated as:

$$Aridity\ Index\ (AI) \quad = \quad \frac{MAP}{MAE}$$

MAP = Mean Annual Precipitation and *MAE* = Mean Annual Potential Evapo-Transpiration.

Mean annual precipitation (MAP) values were obtained from the WorldClim Global Climate Data (Hijmans et al., 2004)*, for years 1950-2000, while PET layers were estimated on the basis of monthly averages by the Global-PET, i.e., modeled using the Hargreaves method* (Hargreaves et al., 1985) *and aggregated to mean annual values (MAE)*[6].

3.7.1 Processing of Aridity dataset

Global-Aridity dataset was downloaded as one grid layer representing the annual average for years 1950-2000. The Aridity Index values reported within the Global-Aridity geodataset had been multiplied by a factor of 10,000 to derive and distribute the data as integers (with 4 decimal accuracy). This multiplier had been used to increase the precision of the variable values without using decimals (real or floating values are less efficient in terms of computing time and space compared to integer values). Global-Aridity values were divided with 10,000 to retrieve the values in the correct units. The global aridity map obtained from CGIAR was for whole world. The data relevant to Pakistan was extracted by a mask of a polygon indicating the borders of Pakistan (Pakistan Admin). The raster map (PakAridityMap) was converted to polygon features, commonly known as "Esri shape file" and subsequently the aridity polygon features for Punjab province (PunjabAridity) was extracted from "PakAridityMap". A higher Aridity Index and darker color represents more humid conditions, while low Aridity Index and lighter colors represent higher aridity. Details about the data format and its processing are described in Appendix C.

[6] http://www.cgiar-csi.org/data/global-aridity-and-pet-database

3.8 Statistical Analyses

Data were stored in a database set up in Microsoft Access. The tables (n = 7) were linked through their primary keys (specific IDs) using the relationship tool. Data entry was controlled with different options in MS Access to avoid mistakes. Screen shots showing lists of tables prepared, the relationship among these tables and details of numeric and text variables in design view are given in Appendix A. All statistical analyses were performed with R for Windows software (version 3.2.1., http://www.r-project.org/) and RStudio as an interface (version 0.99.447, Inc., Boston, MA, USA, https://www.rstudio.com/).

The tick prevalence on animal level was calculated as the number of animals infested with any ticks divided by the total number of animals examined. For the herd prevalence, a farm was considered positive, if at least one animal on the farm was found to carry ticks during the farm visit. The binomial confidence intervals for proportions were estimated using the package 'binom' (Dorai-Raj, 2014) with the 'exact-Clopper-Pearson interval' method. The prevalence of tick-borne pathogens was calculated as the number of infected tick pools divided by total number of tick pools (n = 405). The prevalence of ticks and tick-borne pathogens in agro-ecological zones was compared using the Fisher's exact test for count data. The distribution of tick burdens among animals was not consistent with normality when analysed using the Shapiro-Wilk normality test. Tick burdens in animals in different agro-ecological zones were compared using the Kruskal-Wallis test. Therefore, we assessed the effects of host traits (e.g. gender, breed and species) on tick burden using the Mann-Whitney-Wilcoxon test with continuity correction and the Kruskal-Wallis test. The Tukey and Kramer (Nemenyi) test with the Tukey-Distribution approximation for independent samples was applied in post-hoc analysis in case of animal species and cattle breeds. The effect of the age of the host on the tick burden was evaluated using Spearman's rank correlation.

Additionally, we also assessed the effects of host traits on tick prevalence using a multivariable logistic regression model using the glm() function in R. For this purpose, the animals were categorized as infested or non-infested. We included age, gender and species of the animals as an additive mode of interaction in the model. Age (in years) was used as a numeric variable. The quality of models (goodness of fit, complexity) was compared using the Akaike information criterion (AIC), which is a measure of the relative quality of models and provides a means for model selection.

Data for sheep and goats were merged into one group (called small ruminants) because of the small number of sheep investigated. The effect of breed on tick prevalence was only analysed with univariable analysis and was not included in the multivariable analysis as it only applied to one animal species.

For breed analysis, the smaller groups of goat breeds (Nachi, Dera Din Panah, Desi and Teddy) and sheep breeds (Thalli, Cholistani and Lohi) were merged together and were compared against Beetal or Kajli, respectively. We used the Fisher's exact test for count data to investigate attachment site preferences of each tick species among animal species. For this analysis, we excluded small ruminants and two of the tick species (*Hy. dromedarii* and *Rh. turanicus*) due to low numbers investigated or identified. We examined the co-infestation patterns between the tick species (*Hy. anatolicum* and *Rh. microplus*), their stages (nymphs and adults) and sexes (males and females) using Spearman's rank correlation. The Fisher's exact test for count data was used to test for differences in attachment site preferences among tick species and stages. The *P* value obtained from the Fisher's exact test was adjusted using Bonferroni correction.

The maps were produced in ArcMap software environment v10.3. The base map for Pakistan was obtained from the Database of Global Administrative Areas (GADM) and was set for Gujrat, Gujranwala, Narowal and Okara districts using the 'dissolved function'.

3.8.1 Risk factors study

To measure the effect of various determinants on tick prevalence, a multivariable logistic regression model was built. In the first step, a univariable analysis (Fisher's exact test) was performed to select the variables (predictors), and those, which had produced $P < 0.2$ included with an additive mode of interaction in the multivariable model. Other, more frequently used *P* levels, e.g. 0.05, may fail in identifying variables known to be important (Bursac et al., 2008). Subsequently, the variables were removed from the multivariable model one by one, i.e. using a backward stepwise selection approach, if they were not significant and not a confounder. A variable was considered statistically significant, if the *P* value, for that specific variable, was less than 0.05 and the confounding effect was evaluated by assessing if a change in any remaining parameter estimate was greater than 20% as compared to the full model. For this purpose, the farms were categorized at a dichotomous level (low tick prevalence: 0; high tick prevalence: 1), using a cut-off at 80% prevalence. The ultimate model was fitted with the farm category (low or high

infested) as the response variable. We examined the deviance residuals for homoscedasticity and normal distribution. The Pearson goodness-of-fit statistic (χ^2) was applied to assess the model fits the data. Additionally, AIC values were also utilized to assess the quality of the model. The multivariable model was run using the glm() function. The link function "logit" was used in the model to report the coefficient, the ratio of the coefficient to its standard error and the *P* value. The odds ratio (OR) along with 95% confidence interval (CI) was calculated using the exp() function. The software records an OR of 1 for the reference variable. When the upper limit of the CI of the OR of the examined variable is below than 1, it is considered as a protective factor, and if the lower limit of the CI is above 1, it is considered as a risk factor. ORs are presented for the independent variables that showed statistical significance in the multivariable analysis.

4 Results

4.1 Demographic characteristics of the study population

A total of 471 ruminants (194 buffaloes, 179 cattle, 80 goats and 18 sheep) were examined on 108 livestock farms in two different agro-ecological zones. The median herd size was 10 (8-15) animals. The majority (66%) of the animals were female. The median age of the animals was 2.5 years (Q1-Q3: 1.0-4.5). The median age in large ruminants was 3.0 years (buffalo = 3.1 and cattle = 3.0) and in small ruminants, both in goat and sheep, it was 1.5 years. The median age of the infested animals was 3.0 years (Q1-Q3: 1.0-5.0) while in the non-infested group it was 1.5 years (Q1-Q3: 1.1-2.5).

4.2 Tick prevalence

All the livestock herds, irrespective of their geographic location, were found infested with one or multiple tick species. Within the herds, the tick prevalence varied from 20% to 100% (Mean±SD; 80±20%). The overall proportion of tick-infested ruminants was 78.3% (369/471). It was highest in cattle (89.9%), followed by buffaloes (81.4%), goats (60.0%) and sheep (11.1%) (Table 4.1). The tick prevalence was significantly lower in the semi-arid zone as compared to the arid zone (P = 0.037). Out of all infested animals, 71.0% (n = 265) were infested with *Hy. anatolicum*, 17.0% (n = 59) with *Rh. microplus*, 1.4% (n = 5) with *Hy. dromedarii* and 1.1% (n = 4) with *Rh. turanicus*. 9.5% (n = 36) of the animals were found to have a mixed infestation with more than one tick species.

Table 4.1 Cumulative tick burden, Prevalence and median tick burden in ruminants on livestock farms in the context of agro-ecological zones and districts of Punjab province, Pakistan. The prevalence (OR=0.601, 95% CI 0.37-0.98, *P* = 0.037) and tick burden (*P* = 0.002) were significantly different between the agro-ecological zones

AEZ Districts	NAI/NAO/NTC	Animal species (NAI/NAO)				Tick burden per animal*	Prevalence (95% CI)
		Buffalo	Cattle	Goat	Sheep		
Semi-arid	**100/139/976**	**42/62**	**47/56**	**10/18**	**1/3**	**36 (14-66)**	**72% (64-79)**
ATK	30/43/290	10/17	16/17	04/08	0/1	24 (11-71)	70% (54-83)
GJW	32/47/328	16/21	12/19	04/06	0/1	34 (12-63)	68% (33-81)
KSR	38/49/358	16/24	19/20	02/04	1/1	37 (27-73)	78% (63-88)
Arid	**269/332/2831**	**116/132**	**114/123**	**38/62**	**1/15**	**46 (30-67)**	**81% (76-85)**
OKR	39/55/505	22/24	17/17	00/06	0/8	41 (32-54)	71% (57-82)
KNW	46/51/499	20/22	22/23	04/06	0/0	48 (29-86)	90% (78-97)
MTN[a]	45/51/646	18/20	23/24	04/07	0/0	82 (56-110)	88% (76-96)
VHR	45/58/411	14/20	20/22	10/12	1/4	43 (25-63)	78% (65-87)
BWP	48/57/352	20/22	17/20	11/15	0	34 (27-48)	84% (72-92)
RYK	46/60/418	22/24	15/17	9/16	0/3	42 (28-57)	77% (64-87)
Total	**369/471/3807**	**158/194**	**161/179**	**48/80**	**2/18**	**43 (27-67)**	**78% (74-82)**

AEZ = Agro-ecological zone, NAI = number of animal infested, NAO = number of animal observed, NTC = number of ticks collected, ATK = Attock, GJW = Gujranwala, KSR = Kasur, OKR = Okara, KNW = Khanewal, MTN = Multan, VHR = Vehari, BWP = Bahawalpur, RYK = Rahim Yar Khan

*Values for tick burden are presented as median (1st and 3rd quartiles)

[a]Tick samples were collected at a different time (June 2014)

4.2.1 Effect of host characteristics on tick prevalence

The results of the univariable analysis showed that the tick prevalence was significantly affected by the age ($P < 0.001$), gender ($P < 0.001$), and species ($P < 0.001$) of the animal. The prevalence was significantly different among cattle breeds, where exotic ($P = 0.004$) and crossbred cows ($P = 0.002$) were at higher risk of being infested than indigenous cows. After the univariable analysis, these host traits, except breed, were included in a multivariable logistic regression

analysis, which also showed that the tick prevalence was significantly influenced by the age of the animal ($P = 0.031$) and that older animals were more likely to be infested than younger animals (Table 4.2). The prevalence was higher in females than in males. Among the investigated animal species, cattle had the highest odds of getting infested with ticks, whereas the chances were lower for small ruminants.

Table 4.2 Effect of host characteristics on tick prevalence in various animal species

Variable	Categories	Odds ratio	95% CI	*P* value
Age		1.19	1.02-1.40	0.031
Gender	Male	1		
	Female	2.84	1.64-4.99	< 0.001
Species	Buffalo	1		
	Cattle	2.42	1.30-4.63	0.006
	Small ruminants	0.34	0.19-0.61	< 0.001
Breed*				
Buffalo	Kundi	1		
	Nili Ravi	2.25	0.85-5.64	0.082
Cattle	Indigenous	1		
	Crossbred	6.61	1.75-37.37	0.002
	Exotic	Inf	1.75-Inf	0.004
Goat	Beetal	1		
	Others	1.05	0.37-3.01	1
Sheep	Kajli	1		
	Others	1.26	0.01-111.88	1

*Univariable analysis (Fisher's exact test for count data) was performed to estimate the effect of breed

4.3 Tick burden

The median tick burdens recorded (43 ticks per animal, ranged from 27-67) were significantly different among the animal species ($P < 0.001$). The intensity of infestation was highest in cattle (median = 58), followed by buffaloes (median = 38), goats (median = 19) and sheep (median = 4.5) (Figure 4.1). Large ruminants were more heavily infested than small ruminants ($P < 0.001$) and between bovines, the infestation was higher in cattle ($P < 0.001$) than in buffaloes. Tick burden on

livestock farms was also significantly lower in the semi-arid zone as compared to the arid-zone (P = 0.002) (Table 4.1).

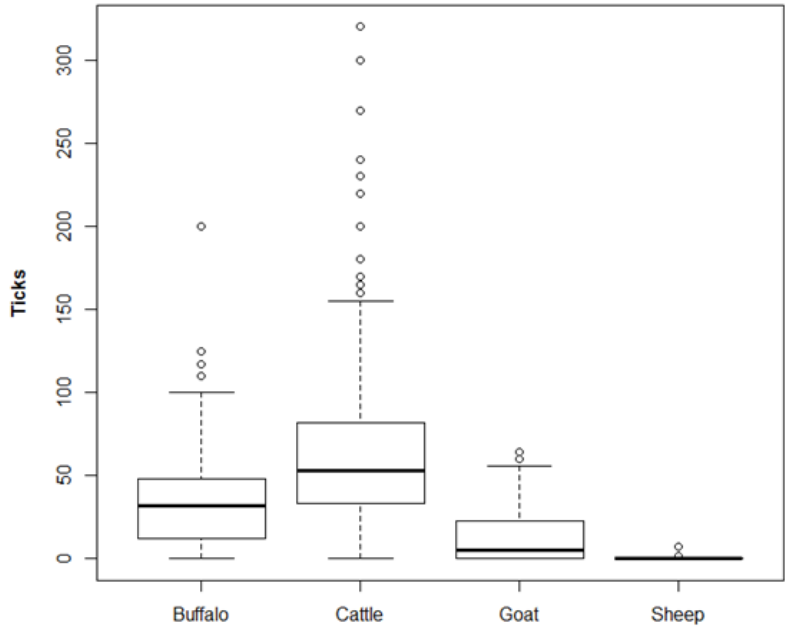


Figure 4.1 Observed tick burden in different animal species

4.3.1 Effect of host characteristics on tick burden

In large ruminants, older animals carried more ticks than younger animals (buffalo, P = 0.020; cattle, P = 0.002), while in small ruminants the difference was not statistically significant (goat, P = 0.680; sheep = 0.988) (Table 4.3). It was observed that female animals had higher tick burdens than male animals (buffalo, P = 0.002; cattle, P < 0.001; goat, P = 0.014; sheep, P = 0.02). The intensity of infestation was significantly different among cattle breeds (χ^2 = 55.42, df = 2, P < 0.001), where indigenous animals had lower tick burdens as compared to exotic (P < 0.001) and crossbred cows (P < 0.001), while the difference was not statistically significant between crossbred and exotic cattle (P = 0.11) (Figure 4.2). In other ruminant species, a statistically significant effect of breed on tick burden could not be demonstrated (buffalo, χ^2 = 2284, P = 0.204; goat, χ^2 = 640, P = 0.627; sheep, χ^2 = 40.5, P = 0.935).

Table 4.3 Effect of host characteristics on tick burden in various animal species

Variable	Statistics	Buffalo	Cattle	Goat	Sheep	Overall
Age	P value	0.02	< 0.001	0.6806	0.988	< 0.001
	Correlation coefficient rho	0.167	0.27	-0.047	-0.003	0.215

Gender	*P* value	0.002	< 0.001	0.014	0.04	< 0.001
	Confidence interval	3.00-22.99	7.99-33.00	0.00-10.69	0.00-10.7	10.99-24.99
	Wilcoxon-statistic	4643	4599	1047	48	32772
Breed	*P* value	0.204	< 0.001	0.628	0.935	NA
	Confidence interval	18.99-1.99	NA	-6.00-1.99	-	NA
	Wilcoxon-statistic	2284	55.42*	640	40.5	NA

NA = Not applicable

*Kruskal-Wallis χ^2 value

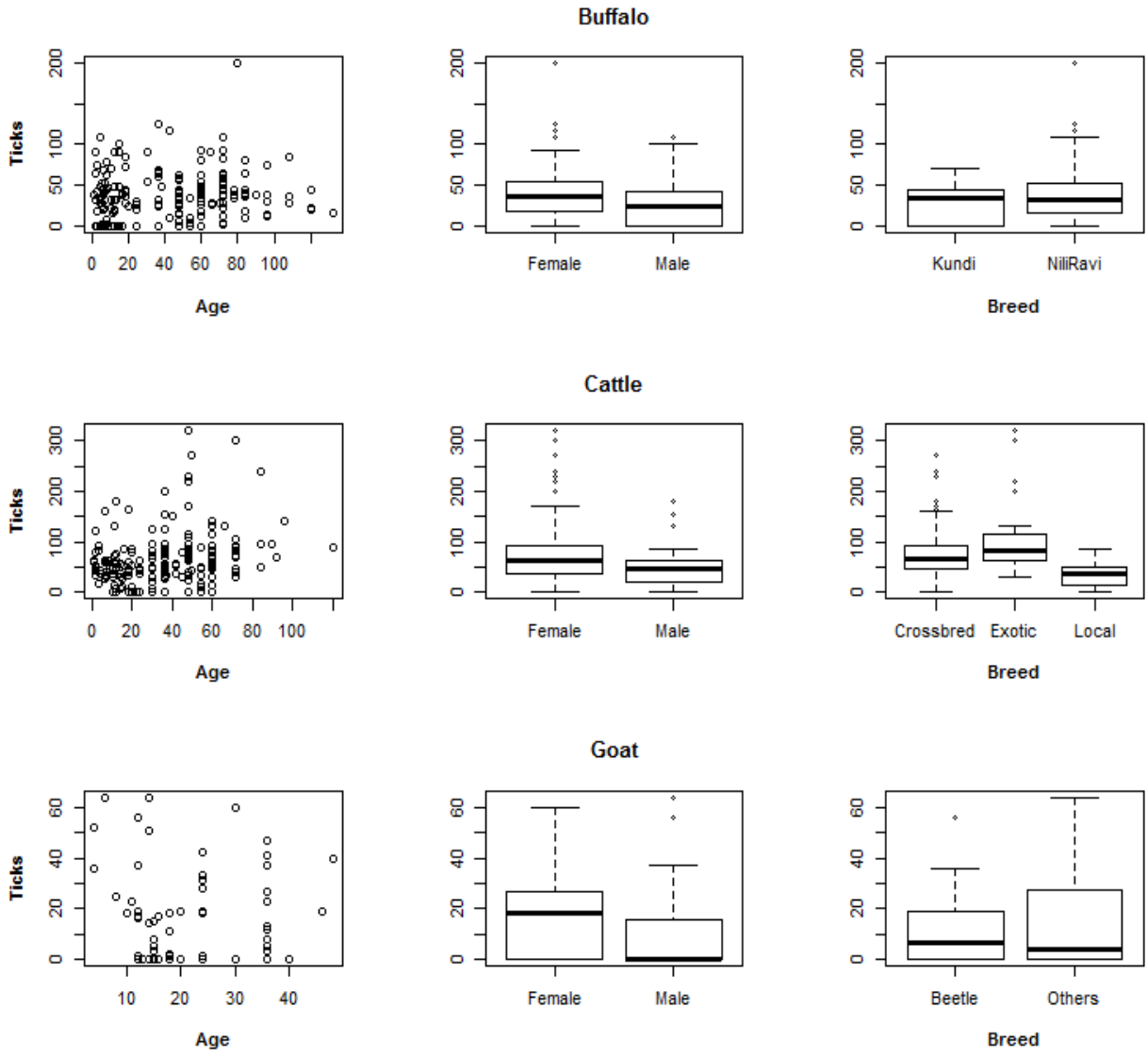


Figure 4.2 Intensity of infestation in animals in relation to their age (in months), gender and breed

4.4 Description of tick species

Ruminants were found infested with four tick species, namely *Hyalomma anatolicum* (*Hy. anatolicum*) (Koch, 1844), *Hyalomma dromedarii* (*Hy. dromedarii*) (Koch, 1844), *Rhipicephalus* (*Boophilus*) *microplus* (*Rh. microplus*) (Canestrini, 1888) and *Rhipicephalus turanicus* (*Rh. turanicus*) (Pomerantsev, 1936) (Figure 4.3 and 4.4). To confirm the findings, fragments of the second internal transcribed spacer (ITS2; ~750 bp) gene from 19 randomly selected samples were sequenced and a BLAST search performed in the NCBI database. Of these 19 sequences, 15 samples, which had previously been morphologically identified as *Hy. anatolicum*, showed 99% identity to strains Xinjiang (accession no. HQ005303) and ha10ffal (accession no. FJ593703) of *Hy. anatolicum* from China and Iran, respectively. Two of the sequences demonstrated 99% identity to a published sequence of *Rh. microplus* isolate Lao1 found in Laos (accession no. KC503276). The sequence of *Hy. dromedarii* showed 97% identity to a registered sequence of *Hy. dromedarii* obtained from a dromedary camel in India (accession no. JQ733570), whereas the sequence of *Rh. turanicus* was 99% identical to the *Rh. turanicus* isolate 80-T-He4 (accession no. KF958417). In addition, a 1,592 bp fragment of the cox1 gene was amplified from five *Rh. microplus* ticks (2 females and 1 male from buffalo, 2 females from cattle) and BLAST results revealed highest identity (96%) with a Chinese *Rh. microplus* isolate from Guizhou, China (accession no. KC503259).

In total, 3,807 ixodid ticks (female: 1303; male: 1261; nymph: 1231; larvae: 12) were collected from 108 livestock farms (Table 4.4). It was observed that *Hy. anatolicum* (n = 3021, 79.3%) was the most common species, followed by *Rh. microplus* (n = 715, 18.8%), *Hy. dromedarii* (n = 41, 1.1%), and *Rh. turanicus* (n = 30, 0.8%).

Rh. microplus (formerly *Boophilus microplus*) was predominant in the semi-arid zone, while *Hy. anatolicum* was the most common tick species in the arid zone. *Hy. dromedarii* and *Rh. turanicus* were only present in the arid zone (Figure 4.5). *Hy. anatolicum* was found in all the districts of the province, while *Rh. microplus* was absent in Multan, Bahawalpur, and Rahim Yar Khan. In all the districts, multiple tick species were found except in Multan district, where only *Hy. anatolicum* was detected. Additionally, *Hy. anatolicum* was found in all life stages on infested animals, while larvae of the other three tick species were not observed.

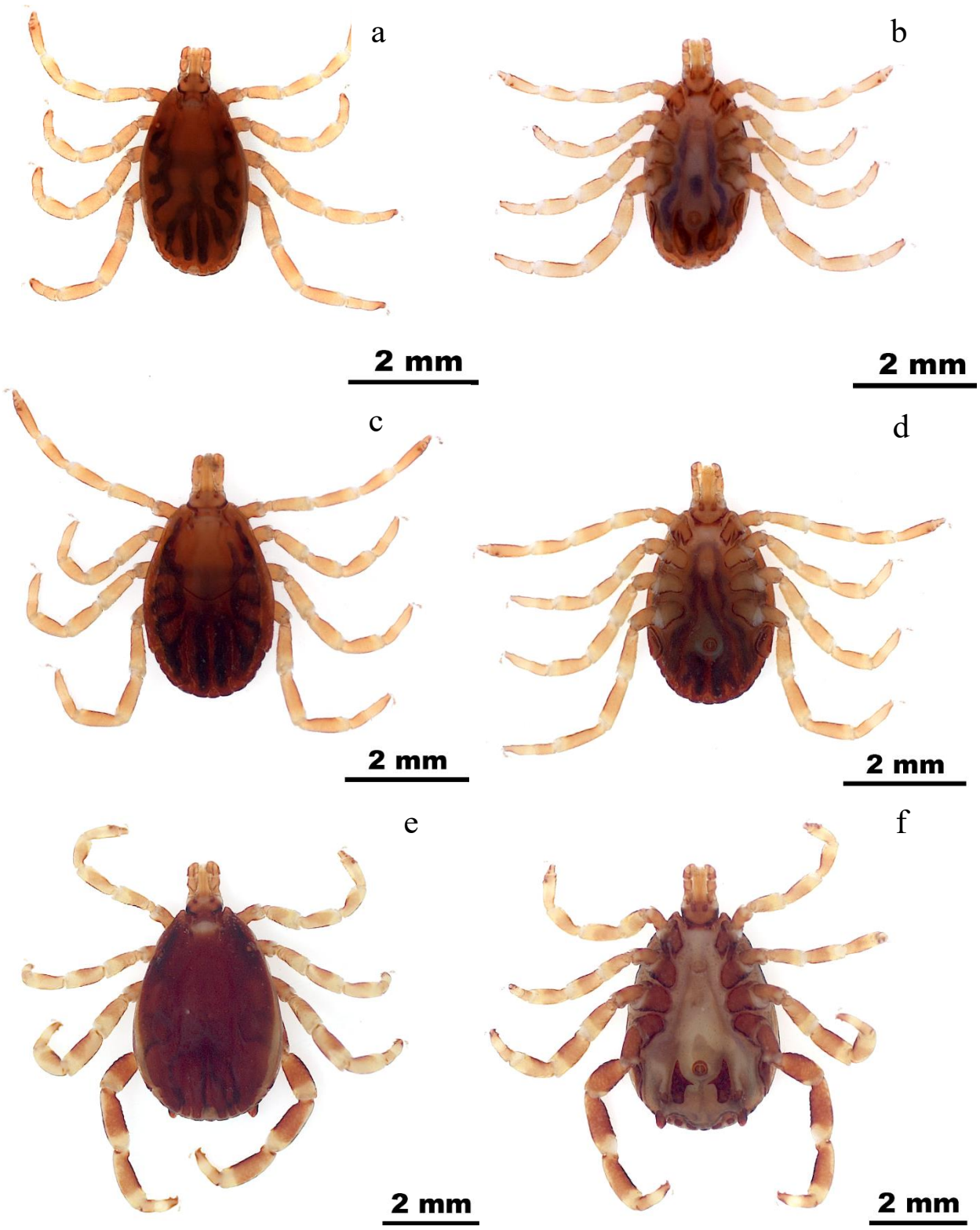


Figure 4.3 *Hyalomma anatolicum* male dorsal view (a), *Hy. anatolicum* male ventral view (b), *Hy. anatolicum* female dorsal view (c), *Hy. anatolicum* female ventral view (d), *Hy. dromedarii* male dorsal view (e), *Hy. dromedarii* male ventral view (f)

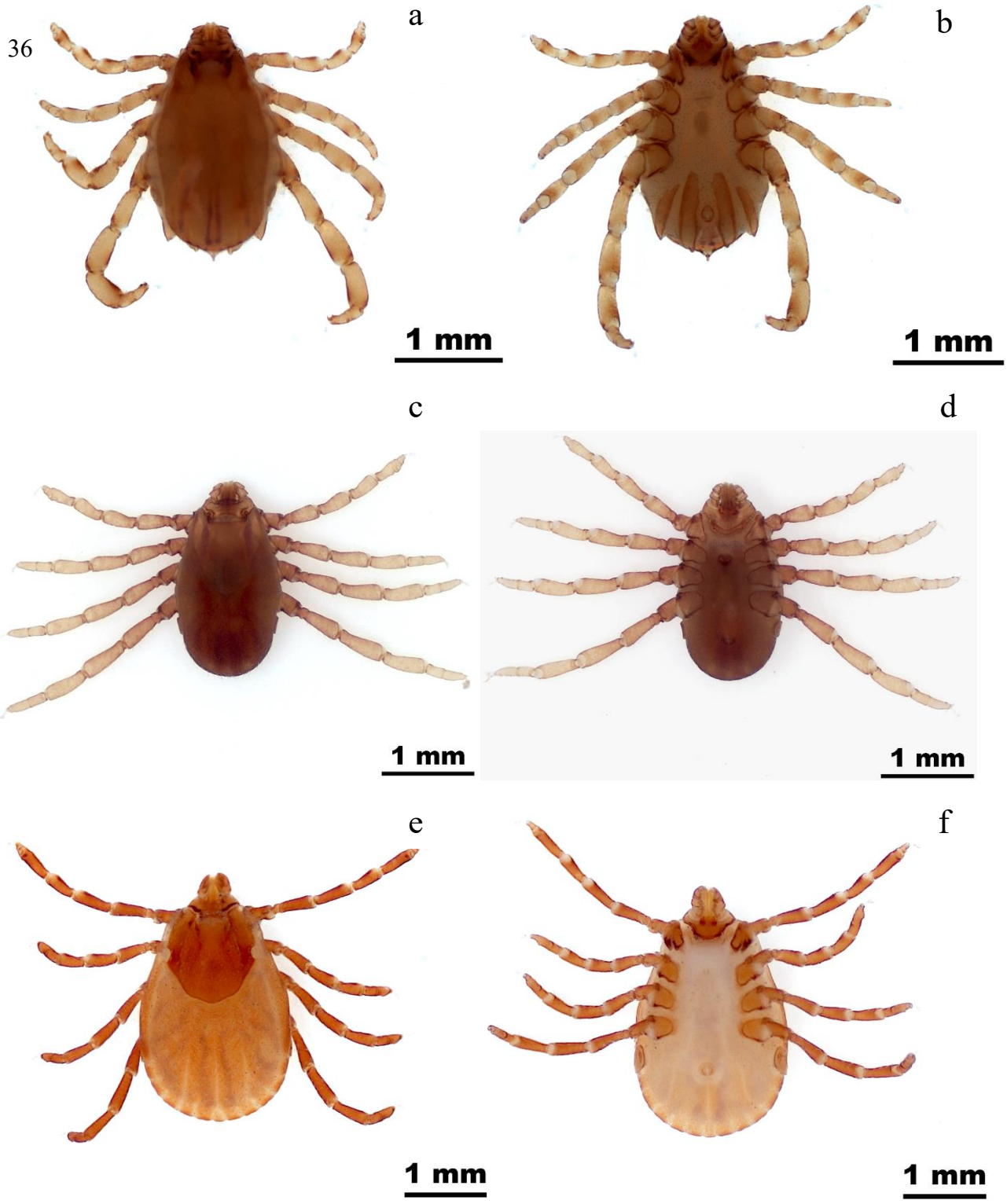


Figure 4.4 *Rhipicephalus microplus* male dorsal view (a), *Rh. microplus* male ventral view (b), *Rh. microplus* female dorsal view (c), *Rh. microplus* female ventral view (d), *Rh. turanicus* female dorsal view (e), *Rh. turanicus* female ventral view (f)

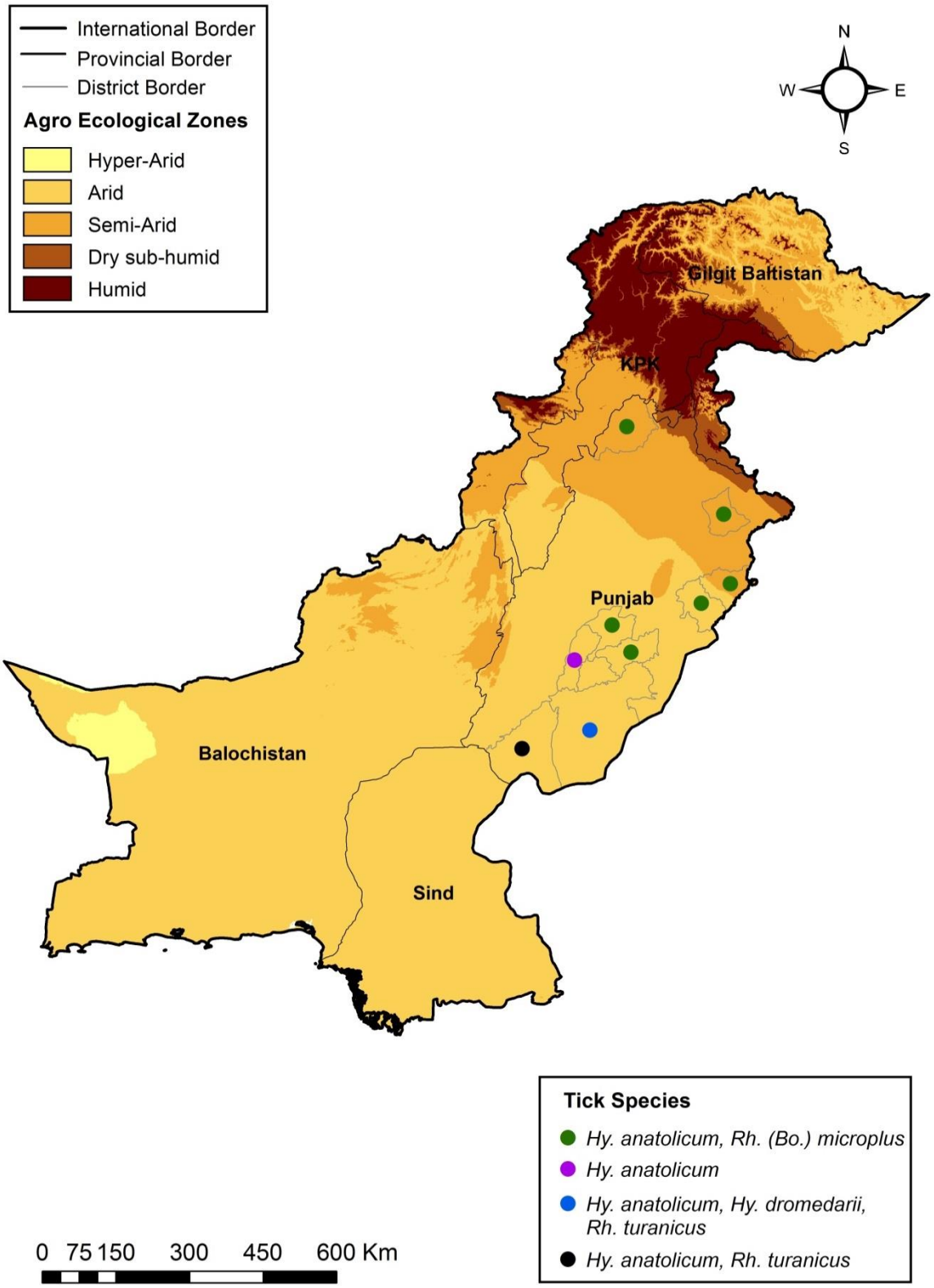


Figure 4.5 Map of Pakistan showing the spatial distribution of tick species in the semi-arid and the arid agro-ecological zone

Table 4.4 Distribution of tick species and their associated host animal species in the semi-arid and the arid agro-ecological zone of Pakistan

AEZ Districts	Hosts	*Hyalomma*		*Rhipicephalus*		Tick species (%)
		anatolicum[b] N/M/F[c]	*dromedarii* N/M/F	*microplus* N/M/F	*turanicus* N/M/F	
Semi-arid						*Hy. anatolicum* = 35.9
ATK	Buffalo	-	-	0/5/16	-	*Rh. microplus* = 64.1
	Cattle	0/25/31	-	10/48/78	-	
	Goat	6/24/24	-	0/10/10	-	
	Sheep	-	-	-	-	
GJW	Buffalo	0/0/3	-	1/33/105	-	
	Cattle	0/7/11	-	7/50/94	-	
	Goat	-	-	0/8/12	-	
	Sheep	-	-	-	-	
KSR	Buffalo	0/55/49	-	9/7/30	-	
	Cattle	7/53/52	-	19/15/50	-	
	Goat	3/0/0	-	3/0/3	-	
	Sheep	-	-	3/0/0	-	
Arid						*Hy. anatolicum* = 94.3
OKR	Buffalo	20/140/84	-	7/1/16	-	*Hy. dromedarii* = 1.5
	Cattle	23/115/65	-	8/1/25	-	*Rh. microplus* = 3.1
	Goat	-	-	-	-	*Rh. turanicus* = 1.1
	Sheep	-	-	-	-	
KNW	Buffalo	72/59/35	-	4/1/4	-	
	Cattle	214/55/27	-	0/2/5	-	
	Goat	21/0/0	-	-	-	
MTN[a]	Buffalo	65/66/96	-	-	-	
	Cattle	168/110/104	-	-	-	
	Goat	26/4/2	-	-	-	
VHR	Buffalo	83/52/47	-	-	-	
	Cattle	85/39/26	-	0/5/10	-	
	Goat	43/12/4	-	-	-	
	Sheep	0/0/2	-	-	-	
BWP	Buffalo	57/65/41	0/4/1	-	0/4/3	
	Cattle	24/25/22	13/16/6	-	-	
	Goat	35/15/9	0/1/0	-	6/0/2	
RYK	Buffalo	71/86/59	-	-	-	
	Cattle	84/31/30	-	-	-	
	Goat	20/12/9	-	-	14/0/1	
	Sheep	-	-	-	-	
Total	**3807**	**1127/1050/832**	**13/21/7**	**71/186/458**	**20/4/6**	
Mean% (95% CI)		**79.3 (78.0-80.6)**	**1.1 (7.7-1.4)**	**18.8 (17.5-20.0)**	**0.8 (0.5-1.1)**	

AEZ = Agro-ecological zone

[a]In MTN (Multan district) the samples were collected at a different time (following year, June) and only *Hy. anatolicum* species was found

[b]Larvae (all belong to *Hy. anatolicum*) are not presented in the table (MTN = 5, VHR = 3, BWP = 3, RYK = 1)

[c]N/M/F: Nymphs/Males/Females

4.5 Screening of ticks for tick-borne pathogens' DNA

A total of 405 (*Hy. anatolicum* = 300, *Rh. microplus* = 89, *Hy. dromerdarii* = 9, *Rh. turanicus* = 7) tick pools (semi-arid zone = 113, arid zone = 292) were screened by RLB assay for the presence of DNA of 41 tick-borne pathogens, i.e. *Anaplasma*, *Ehrlichia, Babesia, Theileria* and *Rickettsia* species. The findings were confirmed by sequencing 35 positive samples. Out of total 405 tick pools, DNA from at least one tick-borne pathogen was found in 148 (36.5%) pools. Among the positive pools, 94 (63.5%) had a mixed infection with two or more (ranging from 2 to 5) tick-borne pathogen species with 18 different combinations, whereas 54 (36.5%) pools were infected with single tick-borne pathogen species. The overall prevalence estimates of tick-borne pathogens in Punjab province were significantly different (χ^2 = 90.2, df = 3, P < 0.001). The prevalence of *Ehrlichia* spp. (22.2%) was highest, followed by *Theileria* (9.9%), *Anaplasma* (7.7%) and *Babesia* spp. (2.5%). Although no statistically significant difference was observed between the semi-arid (37.2%) and the arid zone (36.1%) in the overall prevalence of tick-borne pathogens (χ^2 = 0.01, df = 1, P = 0.91), the prevalence of *Anaplasma* spp. (15.0%) was significantly higher in the semi-arid zone (OR = 3.5, 95% CI = 1.6-8.0, P = 0.001) as compared to the arid zone (4.8%) (Table 4.5). The distribution of tick-borne pathogens in the semi-arid and the arid zone is shown in Figure 4.6.

The overall infection ratio (i.e. the proportion of infected tick pools) of tick-borne pathogens was highest in *Hy. anatolicum* (37.3%), followed by *Rh. microplus* (34.8%), *Rh. turanicus* (28.6%) and *Hy. dromedarii* (22.2%). In the semi-arid zone, the proportion of infected ticks was higher in *Rh. microplus* (38%) than *Hy. anatolicum* (35.7%), whereas in the arid zone, *Hy. anatolicum* ticks were found more often infected (37.6%) than *Rh. microplus* (22.2%). *Hy. anatolicum* ticks were mainly infected with *Ehrlichia* spp. (25.3%), followed by *Theileria* (10.7%), *Anaplasma* (3.7%) and *Babesia* spp. (1.7%), whereas *Rh. microplus* ticks were mainly infected with *Anaplasma* spp. (19.1%), followed by *Ehrlichia* (12.4%), *Theileria* (9.0%) and *Babesia* spp. (5.6%). *Hy. dromedarii* ticks were infected with *Ehrlichia* (33.3%) and *Anaplasma* spp. (11.1%), whereas *Rh. turanicus* ticks were only infected with *Anaplasma* spp. (28.6%). Both, *Hy. dromedarii* and *R. turanicus* ticks were not found infected with *Babesia* or *Theileria* species.

Table 4.5 The overall prevalence of tick-borne pathogens in agro-ecological zones

AEZ Districts	**Tick species**	**NPP/NPT[a]**	***Anaplasma* spp.**	***Ehrlichia* spp.**	***Babesia* spp.**	***Theileria* spp.**	**Prevalence (95% CI)**
Semi-arid		**42/113**	**17***	**20**	**04**	**10**	**37% (28-47)**
ATK	*Hy. anatolicum*	3/11	0	2	0	1	
	Rh. microplus	7/21	1	3	3	2	
GJW	*Hy. anatolicum*	3/3	0	3	0	0	
	Rh. microplus	7/29	6	3	0	1	
KSR	*Hy. anatolicum*	9/28	1	6	0	2	
	Rh. microplus	13/21	9	3	1	4	
Arid		**105/292**	**14**	**70**	**06**	**30**	**36% (31-42)**
OKR	*Hy. anatolicum*	15/39	1	14	0	0	
	Rh. microplus	2/10	1	1	0	0	
KNW	*Hy. anatolicum*	8/43	0	7	0	2	
	Rh. microplus	1/6	0	1	0	1	
MTN	*Hy. anatolicum*	27/45	0	20	2	12	
VHR	*Hy. anatolicum*	13/44	0	7	1	6	
	Rh. microplus	1/2	0	0	1	0	
BWP	*Hy. anatolicum*	18/43	7	9	0	5	
	Hy. dromedarii	2/9	1	1	0	0	
	Rh. turanicus	1/5	1	**0**	0	0	
RYK	*Hy. anatolicum*	16/44	2	10	2	4	
	Rh. turanicus	1/2	1	**0**	0	0	
Total		**147/405**	**31**	**90**	**10**	**40**	**36% (32-41)**

[a]NPP = Number of poles positive, NPT= Number of poles tested

*Fisher's exact test showed significant difference (OR = 3.5, 95% CI = 1.6-8.0, $P = 0.001$) between the two agro-ecological zones

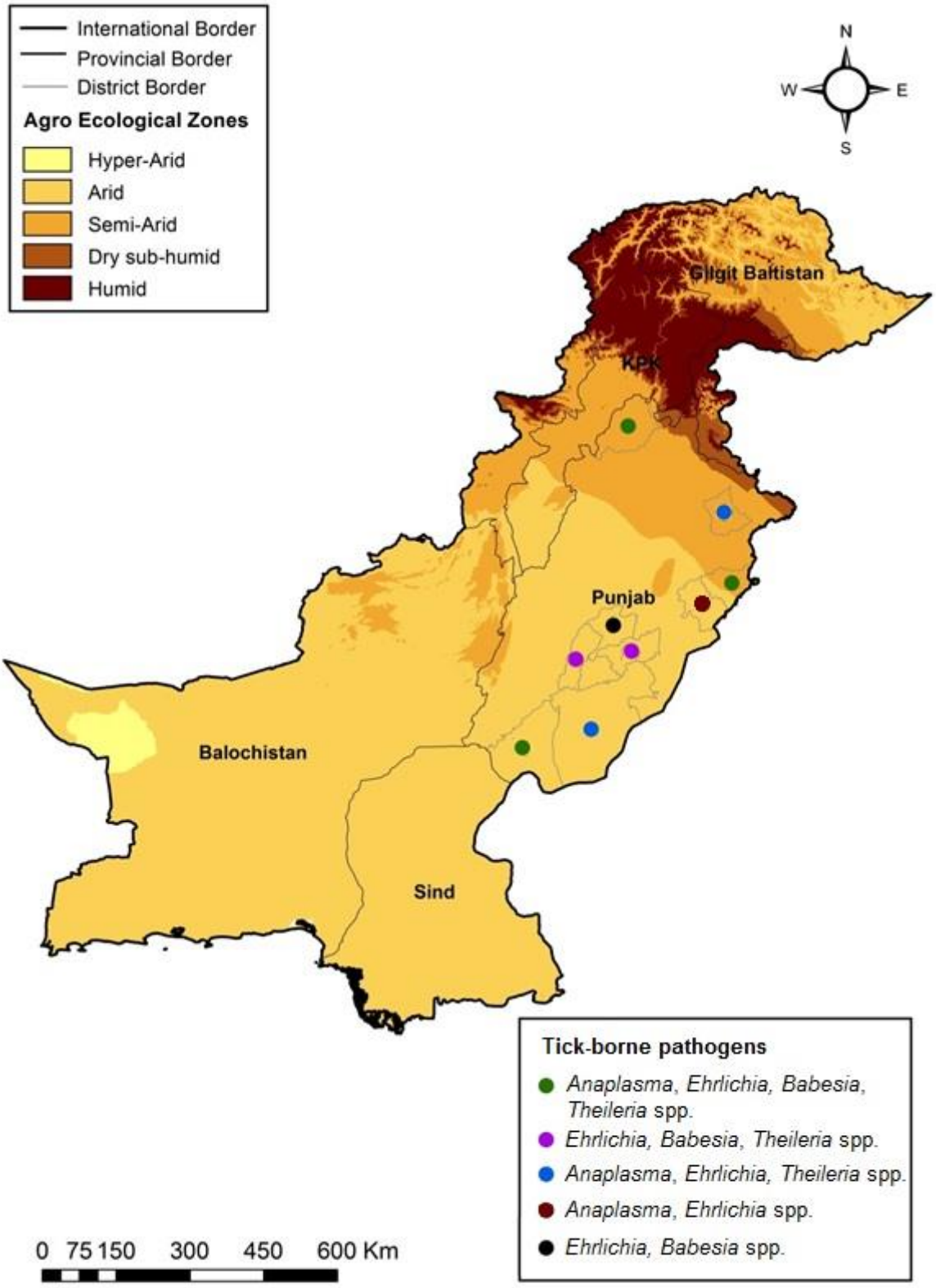


Figure 4.6 Map of Pakistan showing the spatial distribution of the tick-borne pathogens in the semi-arid and the arid agro-ecological zone

The most common found was that of a hitherto uncharacterized species, i.e. *Ehrlichia* sp. (Multan) (18.0%, CI 14.4-22.1), with 99% identity to sequences of the 16S rRNA gene of *Ehrlichia* sp. Firat 3 from Eastern Turkey and *Ehrlichia* sp. BL157-4 from Xinjiang, China (accession nos. EU191229 and KJ410255, respectively). Other common tick-borne pathogens were *Ehrlichia* sp. ERm58 (16.3%, CI 22.8-20.3), *Ehrlichia* sp. Firat (16%, CI 12.6-20), *Theileria annulata* (6.7%, CI 4.4-9.6), *Anaplasma marginale* (5.7%, CI 3.6-8.4), *T. orientalis* (*buffeli*) (3.5%, CI 1.9-5.7), *E. mineirensis* (3.2%, CI 1.7-5.4) and *A. centrale* (2.7%, CI 1.4-4.8). Four of the sequences shared 89 to 99% identity with *E. mineirensis* (previously known as *Ehrlichia* sp. UFMG-EV), a newly characterized *Ehrlichia* species isolated from *Rh. microplus* from Minas Gerais, Brazil (accession no. JX629805). Three sequences showed 98% identity to a published sequence of *Ehrlichia* sp. strain Omatjenne isolated from *Hyalomma truncatum* in the Otjiwarongo district of Namibia (accession no. U54806). Two of the sequences demonstrated 98% identity to *Anaplasma* sp. BL099-6 isolated from *Hyalomma asiaticum* in Xinjiang, China (accession no. KJ410247). Other sequences of *Rickettsiales* (2 from *A. ovis*, 2 from *R. massiliae* and 1 from *R. raoultii*) showed 99% identity to *Anaplasma ovis* strain isolate TC248-1 recovered from *Dermacentor nuttalli* in Xinjiang, China (accession no. KJ410244), *Rickettsia massiliae* MTU5 detected in *Rh. turanicus* tick collected on horses in Camargue, France (accession no. NR074486), and *Rickettsia raoultii* strain Khabarovsk recovered from a *Dermacentor silvarum* tick collected in Russia (accession no. CP010969). *Theileria annulata* ($n = 8$) and *T. ovis* ($n = 1$) sequences were 100% and 99% identical to the sequence of *Theileria annulata* clone Zhangye 27 detected in Bactrian camel blood from Gansu Province, China (accession no. KU554731), and the *Theileria ovis* isolate a5-9mfs recovered from sheep blood from Tehran, Iran (accession no. JN412660), respectively. The *Babesia* spp. sequences ($n = 1$ for *B. occultans*, $n = 3$ for *B. caballi*) showed 99% similarity to *Babesia occultans* isolate HD-127 recovered from *Rh. sanguineus* from Gansu, China (accession no. KT356599), and 89% similarity to the *Babesia caballi* isolate HNXY-H1/2013 recovered from human blood from Shanghai, China (accession no. KJ715182).

The overall prevalence estimates of various *Anaplasma* spp. in Punjab province were significantly different ($P < 0.001$). The prevalence of *A. marginale* (5.7%) was highest, followed by *A. centrale* (2.7%), *Anaplasma* sp. BL099-6 (2.2%), and *A. ovis* (1.5%) (Table 4.6). *A. marginale* and *A. centrale* were mainly found in *Rh. microplus* in the semi-arid zone, whereas in the arid zone both species were mainly harbored by *Hy. anatolicum*. The prevalence estimates of various *Ehrlichia*

species were also significantly different (χ^2 = 159.14, df = 5, P < 0.001). The prevalence of *Ehrlichia* sp. (Multan) (18.0%) was highest, followed by *Ehrlichia* sp. ERm58 (16.3%), *Ehrlichia* sp. Firat (16.0%), *E. mineirensis* (3.2%) and *Ehrlichia* sp. Omatjenne (1.2%). *Ehrlichia* sp. (Multan) (62/73), *Ehrlichia* sp. ERm58 (58/66) and *Ehrlichia* sp. Firat (57/65) were mainly present in *Hy. anatolicum* from both the agro-ecological zones, whereas *E. mineirensis* and *Ehrlichia* sp. Omatjenne were only present in *Hy. anatolicum* ticks from the arid zone. Only four tick pools were found positive for *Rickettsia* spp., of which two were positive for *R. massiliae,* one for *R. raoultii* and one was infected with both *Rickettsia* species.

Table 4.6 Species of *Rickettsiales* bacteria isolated from different tick species in the semi-arid and the arid regions

Tick-borne pathogen species	Semi-arid zone (n=113)		Arid zone (n=292)				Total
	Hy. anatolicum	*Rh. microplus*	*Hy. anatolicum*	*Rh. microplus*	*Hy. dromedarii*	*Rh. turanicus*	
A. centrale	0	2	9	0	0	0	11
A. marginale	1	13	7	1	0	1	23
A. ovis	0	2	3	0	1	0	6
A. sp. BL099-6	1	1	6	0	0	1	9
E. sp. (Multan)	11	8	51	2	1	0	73
E. sp. ERm58	8	5	50	2	1	0	66
E. sp. Firat	8	5	49	2	1	0	65
E. mineirensis	0	0	13	0	0	0	13
E. sp. Omatjenne	0	0	5	0	0	0	5
R. massiliae	1	2	0	0	0	0	3
R. raoultii	0	1	0	1	0	0	2

The overall prevalence estimates of various *Babesia* spp. were not significantly different (P = 0.311), whereas the prevalence estimates of various *Theileria* spp. were significantly different (P < 0.001) in Punjab province (Table 4.7). Among *Theileria* spp., the prevalence of *T. annulata* (6.7%) was highest, followed by *T. orientalis* (3.5%) and *T. ovis* (0.2%). *B. bigemina* and *B. bovis* were present only in *Rh. microplus* ticks from the semi-arid zone, whereas *B. occultans* and *B. caballi* were detected only in *Hy. anatolicum* from the arid zone. *T. annulata* was mainly found (96.2%) in *Hy. anatolicum* ticks.

Table 4.7 *Babesia* and *Theileria* spp. isolated from different tick species in the semi-arid and the arid regions

Tick-borne pathogen species	Semi-arid zone (n=113)		Arid zone (n=292)				Total
	Hy. anatolicum	*Rh. microplus*	*Hy. anatolicum*	*Rh. microplus*	*Hy. dromedarii*	*Rh. turanicus*	
B. bigemina	0	3	0	0	0	0	3
B. bovis	0	1	0	0	0	0	1
B. caballi	0	0	4	1	0	0	5
B. occultans	0	0	1	0	0	0	1
T. annulata	2	0	24	1	0	0	27
T. ovis	0	1	0	0	0	0	1
T. orientalis	1	6	7	0	0	0	14

Hy. anatolicum ticks were mainly infected with *Ehrlichia* sp. (Multan) (20.7%, CI 16.2-25.7), *Ehrlichia* sp. ERm58 (19.3%, CI 15-24.3), *Ehrlichia* sp. Firat (19%, CI 14.7-23.9), *T. annulata* (8.7%, CI 5.7-12.4), *E. mineirensis* (4.3%, CI 2.3-7.3) and *A. centrale* (3%, CI 1.4-5.6). *Rh. microplus* ticks were found infected with *A. marginale* (15.7%, CI 8.9-25), *Ehrlichia* sp. (Multan) (11.2%, CI 5.5-19.7), *Ehrlichia* sp. ERm58 and *Ehrlichia* sp. Firat (7.9% each, CI 3.2-15.5), *T. orientalis* (6.7%, CI 2.5-14.1), and *B. bigemina* (Table 4.8). *Hy. dromedarii* ticks were infected with *A. ovis, Ehrlichia* sp. (Multan), *Ehrlichia* sp. ERm58 and *Ehrlichia* sp. Firat (11.1% each, CI 0.3-48.2), whereas *Rh. turanicus* ticks were infected with *A. marginale* and *Anaplasma* sp. BL099-6 (14.3% each, CI 0.4-57.8).

Table 4.8 Tick-borne pathogen species detected in tick samples collected from different animal species

Tick-borne pathogen species	Buffalo			Cattle			Goat			Sheep
	Hy. anatolicum	*Rh. microplus*	*Rh. turanicus*	*Hy. anatolicum*	*Rh. microplus*	*Hy. dromedarii*	*Hy. anatolicum*	*Rh. microplus*	*Rh. turanicus*	*Hy. anatolicum*
A. centrale	2	0	0	5	0	0	2	2	0	0
A. marginale	1	7	0	7	5	0	0	2	1	0
A. ovis	0	0	0	1	0	1	2	2	0	0
A. sp. BL099-6	5	1	1	2	0	0	0	0	0	0
E. sp. (Multan)	29	7	0	29	2	1	4	1	0	0
E. sp. ERm58	25	3	0	27	3	1	6	1	0	0
E. sp. Firat	25	3	0	27	3	1	5	1	0	0
E. mineirensis	6	0	0	6	0	0	1	0	0	0
E. sp. Omatjenne	1	0	0	4	0	0	0	0	0	0
R. massiliae	0	1	0	0	0	0	1	1	0	0
R. raoultii	0	2	0	0	0	0	0	0	0	0
B. bigemina	0	0	0	0	2	0	0	1	0	0
B. bovis	0	0	0	0	1	0	0	0	0	0
B. caballi	1	0	0	1	1	0	1	0	0	1
B. occultans	1	0	0	0	0	0	0	0	0	0
T. annulata	12	0	0	10	1	0	4	0	0	0
T. ovis	0	0	0	0	0	0	0	1	0	0
T. orientalis	3	3	0	2	1	0	3	2	0	0
Total	**111**	**27**	**1**	**121**	**19**	**4**	**29**	**14**	**1**	**1**

4.6 Risk factors associated with high tick infestation on livestock farms

Tables 4.9 and 4.10 show the results of the descriptive analysis for the categorical and numeric variables, respectively. Only a small proportion of farms (11.1%, 95% CI 5.9-18.6) raised a single ruminant species, while the majority (88.9%, 95% CI 81.4-94.1) held more than one species. Cattle were present on 87.0% (95% CI 79.2-92.7) farms, buffaloes on 92.6% (95% CI 85.9-96.8), goats on 39.8% (95% CI 30.5-49.7) and sheep on 10.2% (95% CI 5.2-17.5).

When the farm owners were asked about the months when ticks occur on their farm, the majority of the farmers (64.8%, 95% CI 55.0-73.8) reported from March to November, while 15.7% (95% CI 9.4-24.0) observed ticks throughout the year and the remaining 19.4% (95% CI 12.5-28.2) did not know. Regarding the months of highest tick infestation, 83.3% (95% CI 74.9-89.8) of the farmers reported from June to September and 16.7% (95% CI 10.2-25.1) did not know. Almost one third of the farmers (29.0%, 95% CI 20.6-38.5) used acaricides to control ticks. When they were asked about the names of the acaricides used, 16 (51.6%, 95% CI 33.1-69.8) out of 31 farmers did not know, whereas the remaining farmers (48.4%, 95% CI 30.2-66.9) used ivermectin (injectable) (19.35%), trichlorfon 97% (topical) (12.90%) or both (16.13%). The majority of the farmers (81.6%, 95% CI 71.0-89.5) used other methods to control ticks like hand-picking, keeping rural poultry, topical application of "Taramira oil" (*Eruca sativa*) on the body of the animals, while only a few farmers (18.4%, 95% CI 10.5-29.0) stated that they had not used anything against ticks.

It was a very common practice that farmers (95.4%, 95% CI 89.5-98.5) used to offer only green roughages to their animals, except for the milking animals, and only 5 farmers (4.6%, 95% CI 1.5-10.5) used to feed all of their animals with green roughages plus concentrates.

Almost half of the farmers (47.2%, 95% CI 37.5-57.1) reported a behavioral change, e.g. restlessness and scratching, in tick infested animals. More than half of the farmers (58.3%, 95% CI 48.5-67.7) had noticed a drop in milk production of the tick infested animals, while the others had not seen (13.0%, 95% CI 7.3-20.8) or did not know (28.7% 95% CI 20.4-38.2) this. Only 2 farmers reported visits of para-veterinary or veterinary staff on a daily basis, while the others reported that these staff visited the farm only when animals became sick (n = 106) or when artificial insemination (n = 65) or vaccination (n = 24) was needed.

Table 4.9 Survey of livestock farms in Punjab province (2013): Summary of categorical variables included in the questionnaire

Variable	Response categories	No. of responses[a] (95% CI)	*P* value[b]
Farm-related variables			
Farm type	Traditional	87.0 (79.2-92.7)	0.601
	Semi-commercial	13.0 (7.3-20.8)	
Rural poultry	Present	40.7 (31.4-50.6)	< 0.001
	Absent	59.3 (49.4-68.6)	
Dogs	Present	36.1 (27.1-45.9)	0.231
	Absent	63.9 (54.1-72.9)	
Purpose	Additional source of income	69.4 (59.8-77.9)	0.0578
	Main source of income	25.0 (17.2-34.3)	0.191
	Own supply	5.6 (2.1-11.7)	
Ruminant species	Single ruminant species	11.1 (5.9-18.6)	0.454
	Multiple ruminant species	88.9 (81.4-94.1)	
Tick control management			
Use of acaricide/s	Yes	29.0 (20.6-38.5)	< 0.001
	No	71.0 (61.5-79.4)	
Animal species treated	All the ruminant species	19.4 (7.5-37.5)	0.995
	Only large ruminants	80.6 (62.5-92.5)	
Method of application	Injection	38.7 (21.8-57.8)	0.995
	Topical	41.9 (24.5-60.9)	0.995
	Injection and Topical	19.4 (7.5-37.5)	
Frequency of application	Once a year	38.7 (21.8-57.8)	0.995
	2-3 times a year	38.7 (21.8-57.8)	
	Don't know	22.6 (9.6-41.1)	
Conventional methods	Do something	81.6 (71.0-89.5)	0.577
	Nothing	18.4 (10.5-29.0)	
Housing			
Housing type	Open	15.7 (9.4-24.0)	0.002

	Traditional rural	84.3 (76.0-90.6)	
Housing material	Hard bricks with wood/iron	73.1 (63.8-81.2)	
	Soft bricks with wood/straw	16.7 (10.2-25.1)	0.931
	Soft and Hard bricks	10.2 (5.2-17.5)	0.260
Floor type	Soft	52.8 (42.9-62.5)	0.033
	Hard	47.2 (37.7-57.1)	
Feeding method	Stall feeding – zero-grazing	75.9 (66.7-83.6)	0.002
	Grazing	24.1 (16.4-33.3)	
Feed storage	Yes	57.4 (47.5-66.9)	0.243
	No	42.6 (33.1-52.5)	
Boundary wall	Yes	70.4 (60.8-78.8)	0.975
	No/Incomplete	29.6 (21.2-39.2)	
Trees	Present	89.8 (82.5-94.8)	0.261
	Absent	10.2 (5.2-17.5)	
Frequency of removal of animal dung	Daily basis	43.5 (34.0-53.4)	0.031
	After a long time (monthly basis)	56.5 (46.6-66.0)	

[a]No. of responses = $\frac{Number\ of\ responses\ for\ specific\ category}{Total\ number\ of\ respondents}\ X\ 100$

[b]Probability values of Univariable analysis using Fishers' exact test

Table 4.10 Survey of livestock farms in Punjab province (2013): Summary of numeric variables included in the questionnaire

Variable	Median (95% CI)	*P* value
Farm size in hectares	0.06 (0.04-0.07)	0.756
Herd size	10.0 (8.5-11.5)	0.846
Distance to nearest livestock farm	219.3 (182.4-256.2)	0.087

4.6.1 Variables included in the multivariable logistic regression model

To identify the potential risk factors associated with high tick prevalence on livestock farms a multivariable logistic regression model was built. Initially, the model was run with eight variables which had produced $P < 0.2$ in univariable analysis (Table 4.11). This model had an AIC of 109.02.

Afterwards, a backward stepwise selection approach was applied and the variables were removed one by one if they were not significant and not a confounder. The final model was fitted with the farm category (low or high tick prevalence) as the response variable, whereas the explanatory variables (predictors) were presence/absence of rural poultry, use of acaricides, housing type and feeding method. The results of statistically significant variables, which were included in the final logistic regression model, are presented in Table 4.12. This model had an AIC of 102.0. The Pearson goodness-of-fit statistic showed that the model adequately fits the data ($P = 0.748$). The presence of rural poultry on farm significantly ($P = 0.006$) affected the tick prevalence and the odds of getting higher tick prevalence on farms where rural poultry was absent were 4.4 times as high as on farms with rural poultry. The tick prevalence was significantly lower ($P < 0.001$, OR = 7.5) on farms where acaricides had been used. The housing type had also a significant effect on the tick prevalence ($P = 0.007$) and the chances of getting higher tick prevalence on farms with traditional rural housing system were almost 13 times as high as on farms with open houses. The feeding method was also an important variable associated with the tick prevalence and farms, where grazing was practiced, had a higher prevalence ($P = 0.003$, OR = 12.6) as compared to farms with a stall feeding system.

Table 4.11 Survey of livestock farms in Punjab province (2013): Summary of variables included initially in the multivariable logistic regression model

Variable	Response categories	Odds ratio	95% CI	*P* value
Rural poultry	Present	1		
	Absent	4.5	1.5-14.1	0.008
Purpose of farming	Main source of income	1		
	Additional source of income	1.5	0.5-4.6	0.483
Use of acaricide/s	Yes	1		
	No	7.6	2.3-29.7	0.001
Housing type	Open	1		
	Traditional rural	12.4	2.1-121.6	0.012
Floor type	Soft	1		
	Hard	1.0	0.3-3.2	0.958
Feeding method	Stall feeding	1		
	Grazing	12.8	2.9-98.8	0.003

Frequency of removal of animal dung	After a long time (monthly basis)	1		
	Daily basis	0.7	0.2-2.4	0.592
Distance to nearest livestock farm	> 219 m	1		
	< 219 m	0.7	0.2-2.1	0.513

Table 4.12 Survey of livestock farms in Punjab province (2013): Summary of variables included in the final multivariable logistic regression model

Variable	Response categories	Odds ratio	95% CI	*P* value
Rural poultry	Present	1		
	Absent	4.4	1.6-13.0	0.006
Use of acaricide/s	Yes	1		
	No	7.5	2.4-26.7	< 0.001
Housing type	Open	1		
	Traditional rural	13.1	2.4-118.0	0.007
Feeding method	Stall feeding	1		
	Grazing	12.6	2.9-96.4	0.003

4.7 Attachment site preferences

The distribution of tick species, their stages and sexes were noticeably different among host body areas (Table 4.13). Attachment site preferences significantly varied by animal species for both tick species (*Hy. anatolicum*: $\chi^2 = 140.4$, $P < 0.001$; *Rh. microplus*: $\chi^2 = 77.6$, $P < 0.001$). Immature ticks of both species (*Hy. anatolicum* and *Rh. microplus*) were observed in the highest numbers, both in buffalo and cattle, in the ear region. However, immature ticks of *Rh. microplus* were exclusively absent in the tail region of both the animal species (Figure 4.7a to d). Adults of both the tick species were found in increasing order of abundance in the ear, brisket, udder and tail regions in buffalo (Figure 4.7a & b). Distribution patterns of adults of both tick species were slightly different in cattle. Adults of *Hy. anatolicum* were found in increasing order of abundance in the ear, tail, brisket, and udder regions, whereas adults of *Rh. microplus* in the ear, tail, udder and brisket regions. Both tick species co-occurred in all the regions of buffalo and cattle. In buffalo, the brisket region was least infested, whereas in cattle the tail region harbored a quite low tick loads.

Table 4.13 Attachment site preferences between adults and nymphs of the tick species compared using Fisher's exact test for count data

Tick species	Statistics	Comparisons between predilection sites					
		Ear & Brisket	Ear & Udder	Ear & Tail	Brisket & Udder	Brisket & Tail	Udder & Tail
Hy. anatolicum	*P* value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.003
	OR	12	0.02	0.04	0.31	0.48	0.65
	95% CI	8.6-17	0.02-0.03	0.03-0.06	0.23-0.42	0.35-0.64	0.49-0.8
Rh. microplus	*P* value	< 0.001	< 0.001	< 0.001	0.309	0.032	0.332
	OR	24.9	0.08	0.17	2.1	4.1	0.50
	95% CI	7.2-113	0.02-0.27	0.04-0.53	0.43-10.8	0.92-21.2	0.11-2.1

OR = Odds ratio; CI = Confidence interval
Bonferroni correction was used to calculate the critical *P* value, i.e. 0.008

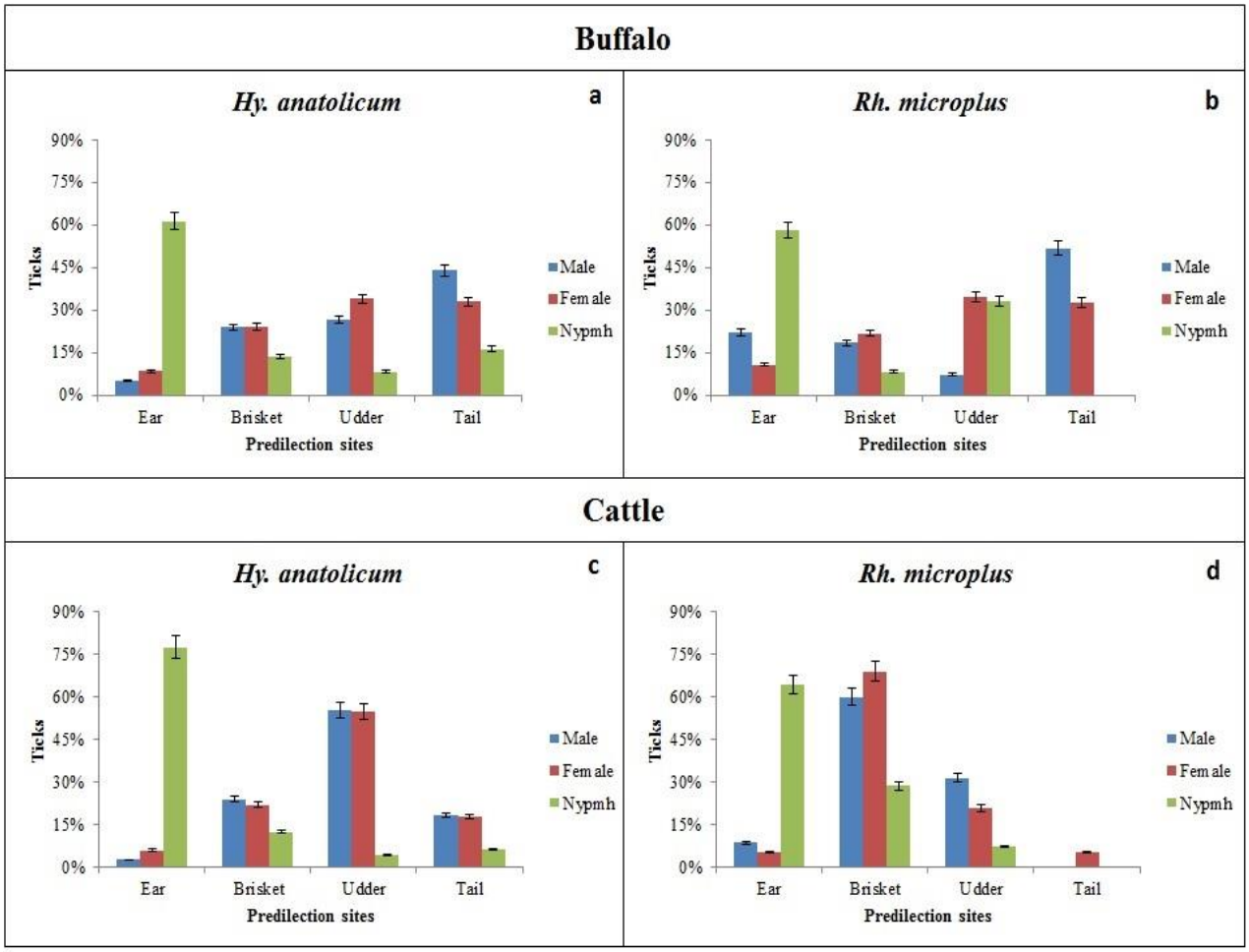


Figure 4.7 Attachment site preferences of male, female and nymph of *Hy. anatolicum* and *Rh. microplus* in buffaloes (a & b) and cattle (c & d)

5 Discussion

Ticks are economically the most important pests of cattle and other domestic species in tropical and subtropical countries (Muhammad et al., 2008). Ticks and TBDs have a huge impact on animal as well as human health. Ticks affect animal health mainly through: (i) biting stress that leads to loss of production, (ii) physical damage, (iii) poisoning, and (iv) transmission of pathogens including protozoa, bacteria and viruses (Jabbar et al., 2015). Although, economic losses related to ticks are mainly due to the diseases which they transmit (Garcia, 2003), financial losses associated with nagging irritation and depreciation of the value of skins and hides (up to 20-30%) are also significant (Biswas, 2003). We conducted a cross-sectional study to assess the species diversity of ticks and their burdens on ruminants and the pathogens they carried in two agro-ecological zones of Pakistan.

5.1 Tick prevalence

5.1.1 Tick prevalence in animal species

We examined 471 animals (194 buffaloes, 179 cattle, 80 goats and 18 sheep) on 108 livestock farms in nine districts of Punjab province. The sample size was calculated assuming 50% tick prevalence, which means that half of the livestock population (small and large ruminants) in the province could be infested with ticks, to ensure maximum precision of the respective prevalence estimates. Among the selected group, the majority (66%) of the animals were female. The greater number of females and adults rather than males and younger animals sampled reflects the gender and age composition of livestock herds in Punjab province (Anonymous, 2006).

In our study, the overall estimated tick prevalence in ruminants was 78.3% (369/471). A number of studies have been conducted in different geographical areas of Pakistan to investigate the tick prevalence in ruminants. However, there is a great variation in tick prevalence estimates in Pakistan and the neighbouring countries as well. Reported figures vary from 31% (A. Iqbal et al., 2013) to 85% (Mustafa et al., 2014) in Pakistan, up to 77% in Iran (Sofizadeh et al., 2014) and 58% in India (Singh and Rath, 2013). This variation in tick prevalence could be mainly due to the difference in geographical and climatic conditions of the study areas, study seasons, target populations and husbandry practices (Iqbal et al., 2014). Tick prevalence in ruminants is much

higher in Asia and Africa as compared to the other continents (Sajid et al., 2011). Plausible reasons of this high tick infestation could be the warmer climate (temperature is a well-known stimulator for tick development), difference in housing types, husbandry practices and tick control strategies. Notably, the prevalence of ticks in the Punjab province has been increasing rapidly for last few years, which could be partially attributable to acaricidal resistance (Ali et al., 2013; Mustafa et al., 2014; Sajid et al., 2009, 2008; Tasawar et al., 2014). This phenomenon has been documented in other countries (Abbas et al., 2014), but no study has yet evaluated acaricidal resistance in Pakistan (Jabbar et al., 2015). We found that the prevalence was significantly higher ($P = 0.037$) in the arid zone (81%), where the daily temperature is higher and provides optimal conditions for tick multiplication, than in the semi-arid zone (72%).

It is evident from the results that the tick prevalence was significantly different among animal species, which concurs with previous studies (Ghosh et al., 2007; Sajid et al., 2008). We observed that in bovines the prevalence was higher in cattle than buffaloes, which is also in accordance with previous studies (Rehman et al. 2004; Sajid et al. 2009; Ali et al. 2013; Chhillar, Chhilar, and Kaur 2014). The higher tick prevalence in cattle as compared to buffaloes might be linked with the drier habitats and thinner skin of cattle as compared to the marshy habitats and thicker skin of buffaloes (Sajid et al., 2009), and host genetics may also play a role (Jonsson et al., 2014). The tick prevalence found in buffaloes (81.4%) is in agreement with a recent report from Punjab province (Mustafa et al., 2014), who estimated 84.3% prevalence, but is high in comparison to other studies (Sajid et al. 2008; Sajid et al. 2009; Tasawar et al. 2014), who reported 40.1%, 47.3% and 52.5%, respectively. The high tick prevalence determined in a recent study may indicate a prevalence increase in buffaloes, which could be attributable to the significantly decreased number of marshy places as they are assumed to be an important source for the breeding of mosquitoes and spread of important diseases like malaria, which is endemic in Pakistan (Zubairi et al., 2013). These new conditions may have forced buffaloes to spend more time during the day on dry land rather than in water, and consequently become more and more exposed to tick infestation.

Limited information is available about the tick prevalence in small ruminants in Pakistan, as only a few studies have focused on these animals. In general, the tick prevalence observed in the present study was higher in goats as compared to sheep, which is also in agreement with a local study (Sajid et al., 2008). Although a particular reason for lower tick prevalence in sheep is not evident, one could speculate that the hairy wool might be an important protective factor against tick

infestation (Sajid et al., 2008). The tick prevalence in goats was estimated to be 60%, which is completely in line with a local study (Sajid et al., 2011). Another study observed a slightly lower tick prevalence of 41.5% (Irshad et al., 2010). However, the latter study was conducted in the northern part of the province, where we also observed a low prevalence. Tick infestation was lowest (11.1%) in sheep, which partially coincides with a previous study from the same geographical region, where authors examined 1400 sheep and found that no animal was infested with ticks (Sajid et al., 2008). Another study from the northern part of the province reported a tick prevalence of 43.4% in sheep (Irshad et al., 2010). However, the results of the tick prevalence in sheep cannot be compared and extrapolated because of the small number of subjects in our study.

5.1.2 Tick prevalence in agro-ecological zones

The present study revealed that all the livestock herds were found infested with one or multiple tick species. Within-herd tick prevalence ranged from 20% to 100% (Mean±SD = 80±20%). These results are not comparable to previous studies from Pakistan as no one reported within-herd tick prevalence. The tick prevalence on the animal level was lower in the semi-arid zone as compared to the arid zone. Likewise, the median tick burden in animal species was also lower in the semi-arid zone than the arid zone. The semi-arid zone is located at a higher elevation and observes low annual mean temperatures (Min. and Max.) as compared to the arid zone (Table 3.1). Tick activity is delayed by higher altitude and lower temperatures and vice versa (Jouda et al., 2004; Perret et al., 2000). Previous reports from Pakistan did not consider the agro-ecological zones and were based on administrative units only (Jabbar et al., 2015). Nevertheless, fluctuations in tick prevalence have been reported in different areas of the same region (A. Iqbal et al., 2013). A study from the lower Punjab reported significant difference in tick prevalence between the two districts and even within the districts among tehsils (Sajid et al., 2009). Moreover, variations in tick prevalence in buffaloes of different geographical conditions have previously been documented in Pakistan (Khan et al., 1993; Ramzan et al., 2008; Sajid et al., 2009). However, the variations in tick prevalence within the same geographical region can be attributed to differences in husbandry practices including tick control strategies and awareness of the farmers (Ghosh et al., 2007).

5.2 Tick burden

Like the tick prevalence, tick burden (also known as intensity of tick infestation) was also highest in cattle followed by buffaloes, goats and sheep. Our results are in agreement with a previous study from Pakistan (Ali et al., 2013). These authors reported a higher intensity of tick infestation in

cattle as compared to buffaloes, but they did not mention the average tick burden. In the past, studies have shown that the buffalo was a less suitable host than cattle for *Rh. microplus* ticks, the second most common tick species in our samples. A plausible explanation could be that the thick skin of the buffalo reduced the ability of these ticks to attach because of their short hypostome. Additionally, the immune system of the buffalo showed an increased sensitivity against tick proteins than that of cattle (Benitez et al., 2012). The median tick burden (43) was higher as compared to a previous study (28) from Tanzania (Swai et al., 2005) in spite of the practice of hand removal of ticks practiced by most of the rural farmers. This difference in tick burdens might be due to the frequent use of acaricides in the latter study.

5.3 Effect of host characteristics on tick infestation

A number of host related factors including age, sex, breed, lactation stage, innate immunity, nutritional status of the animal, and body condition can affect tick infestation, (Anderson et al., 2013). Although host characteristics are frequently thought to explain the intensity of tick infestation, the spatio-temporal distribution of ticks during their questing stages may perhaps have a leading effect on the clustering of ticks on the host.

5.3.1 Gender

Our finding that tick burdens were higher in female animals as compared to males is consistent with previous studies carried out in ruminants (Asmaa et al., 2014; A. Iqbal et al., 2013; Kabir et al., 2011; Sajid et al., 2011). Male animals are mainly used for draught and sacrificial purposes on the celebration of "Eid-ul-Adha" (the biggest sacrifice and holy festival of Muslims) and for breeding purposes. Therefore, they receive more attention, like frequent grooming including the manual removal of ticks, which would result in low tick burdens. It is pertinent to mention that the sacrifice festival was within the time limit of the study, so that this could explain the low number of sheep and goats sampled. A few weeks before the festival, animals, mainly goat and sheep, are transported for sale from the farms to animal markets in the big cities. It has been postulated that both, pregnancy and lactation stress, decrease the resistance in females (Sutherst et al., 1983; Utech et al., 1978). Recently, a study reported higher tick burdens in pregnant buffaloes (Anderson et al., 2013) as compared to non-pregnant animals. Downregulation of the immune response, which may explain a higher tick burden, has been observed in pregnant animals as a result of trade-offs among these energetically expensive life history functions (French et al., 2007; Friedl and Edler, 2005) or to care for the growing fetus (Tizard, 2009).

5.3.2 Age

A significantly lower tick burden in buffalo and cattle calves as compared to older animals is in agreement with previous studies in buffaloes (Anderson et al., 2013), cattle (Jongejan et al., 1987; Lorusso et al., 2013; Marufu et al., 2011; Okello-Onen et al., 1999; Tessema and Gashaw, 2010; Tiki and Addis, 2011; Yakhchali and Hasanzadehzarza, 2004) and roe deer (Vor et al., 2010). A previous study estimated that adult cattle had a higher chance of carrying ticks (OR = 12.3) than calves (Swai et al., 2005). The lower tick burdens recorded in calves could indeed be due to a combination of factors, including the frequent grooming of calves, especially head, ears and neck regions, by their dams (Fivaz and de Waal, 1993; Okello-Onen et al., 1999) and the smaller surface area of younger animals as compared to adults (Mooring et al., 2000). It could be reasonably assumed, that animals with a larger body surface would definitely provide more contact opportunities for ticks to attach themselves (Anderson et al., 2013). This can also be explained by the body size principle, i.e. the smaller the animal, the fewer ticks it can afford to aggregate per unit area of body (Mooring et al., 2000). Furthermore, young animals are more capable of protecting themselves from ticks by innate and cell mediated immunity (Okello-Onen et al., 1999; Wikel and Bergman, 1997), although it has to be stressed that we did not evaluate the immune status of the animals in our study.

5.3.3 Breed

Our result that tick infestation was highest in exotic cattle (Taurine cattle or *Bos taurus taurus*) followed by crossbred and indigenous cows (Zebu cattle or *Bos taurus indicus*) is in line with a previous study from Pakistan (Ahmed et al., 2012; Sajid et al., 2009), in which a similar pattern among cattle breeds was reported. Higher tick infestation in exotic breed as compared to indigenous cattle has also been reported in Argentina (Mangold et al., 1986), Ethiopia (Solomon and Kaaya, 1996) and Egypt (Asmaa et al., 2014). Resistance to one-host ticks, e.g. *Rh. microplus*, is related to the proportion of zebu (*Bos indicus*) genes in the breed (Brizuela et al., 1996; Rechav and Kostrzewski, 1991). It is well known that the host resistance to tick infestations is a genetically determined trait (Jonsson et al., 2014). In *B. indicus* and their crosses, the resistance against different tick species has been found a highly heritable trait (Vercoe and Frisch, 1986). Although, the mechanism of resistance acquired by the indigenous breeds is not fully understood, it could be related to pre-immunity against ectoparasites, which often established through frequent contacts with the parasites in early stage in life (Ahmed et al., 2012). Differences in the immune responses

among cattle breeds has been observed, which might play an important role in the development of tick resistance (Fivaz et al., 1991). Moreover, histamine stimulated grooming by the host is responsible for tick removal (Kaufman, 1989) and higher concentrations of histamine are measured in cattle that are resistant to ticks as compared to non-resistant cattle (Willadsen, 1980). However, plausible factors, which affect the breed susceptibility for tick infestation still need to be explored in indigenous cattle breeds of Pakistan. Keeping the above facts in mind, host-tick resistance can be exploited successfully in cattle breeding programmes as a means, which could contribute to the biological control of tick infestation (Ahmed et al., 2012).

In experimental studies, crosses of Sahiwal cattle with Ankole breed in Burundi (Moran et al., 1996) and with Friesian and British animals in Australia (Nicol et al., 1982) proved high level of tick resistance and hence low tick burdens. The Sahiwal breed is said to exhibit a high degree of resistance to ticks (ASS, 2016; FAO, 2004) as the skin naturally keeps on moving and does not allow ticks to attach themselves (Anonymous, 2016). Furthermore, the Sahiwal breed is more resistant than *Bos taurus* dairy breeds to TBDs as well. A previous study compared responses to experimental infection with *T. annulata* in Sahiwal and Holstein calves, in which all the calves of Sahiwal breed, contrary to Holstein calves, survived without treatment (Glass et al., 2005).

In tropical and subtropical Australia, the integrated pest management (IPM) approach to tick control is now applied in the dairy industry with the development of adapted and more resistant crossbreeds such as the Australian Friesian-Sahiwal (AFS) and the Australian Milking Zebu (AMZ), which is developed with the crosses of Jersey with Sahiwal and Red Sindhi. In Kenya, the International Livestock Centre for Africa (ILCA) has demonstrated the suitability of Ayrshire-Sahiwal crosses. However, an often heard drawback of selective breeding for tick resistance is that resistant breeds typically have poor production characteristics (growth, milk yield) compared to European breeds (which have been selected for a high production).

We found that tick infestation in buffalo breeds was not significantly different, which is consistent with previous studies (A. Iqbal et al., 2013; Sajid et al., 2009). The possible explanation could be that both breeds (Nili-Ravi and Kundi) were local to the study area.

5.4 Description of identified tick species

In total, 3,807 (12 larvae, 1,231 nymphs, 1,303 females and 1,261 males) ixodid ticks representing four different species were collected: *Hyalomma anatolicum* (n = 3,021, 79.3%), *Rhipicephalus*

microplus (*n* = 715, 18.8%), *Hyalomma dromedarii* (*n* = 41, 1.0%), and *Rhipicephalus turanicus* (*n* = 30, 0.9%). To confirm the morphological identification, fragments from the ITS2 gene were amplified and subsequently sequenced from 19 tick specimens. Fukunaga et al (2000) performed the phylogenetic analyses of Ixodidae ticks using the ITS2 gene and found it as a good marker (Abdigoudarzi et al., 2011). In addition, a 1592 bp fragment of the cox1 gene was amplified from five *Rh. microplus* ticks and BLAST results indicated highest identity (96%) with a Chinese *Rh. microplus* isolate from Guizhou.

This is the first report from Pakistan, which confirmed the identified tick species using molecular techniques, which should be utilized to identify and discriminate the different species and subspecies of ticks, if the morphological identification is doubtful or ambiguous (Abdigoudarzi et al., 2011). Previous studies from the country were only based on morphological identification (Ahmed et al., 2012; Ali et al., 2013; Iqbal et al., 2014; Sajid et al., 2011) and the majority of them identified tick samples at the genus level (Ahmad et al., 2014; Durrani et al., 2008; Durrani and Kamal, 2008; Irshad et al., 2010; Tasawar et al., 2014). However, the identified tick species have previously been reported in ruminants from Punjab province (Ali et al., 2013; Iqbal et al., 2014; Sajid et al., 2011) with the exception of *Rh. turanicus*.

Overall, *Hy. anatolicum* was the most abundant tick species found in this study. It parasitized all the animal species in both the agro-ecological zones. These findings are in agreement with previous reports from Pakistan (Ali et al., 2013; Iqbal et al., 2014; Sajid et al., 2011; Sultana et al., 2015). A number of studies from Pakistan (Ali et al., 2013; Atif et al., 2012; Sultana et al., 2015) and neighbouring countries such as Iran (Ganjali et al., 2014; Nasiri et al., 2010; Salim Abadi et al., 2010; Shemshad et al., 2012) and India (Chhillar et al., 2014) also reported *Hyalomma* spp. as the most common tick species. In all districts, multiple tick species were found except Multan district, where only *Hy. anatolicum* was detected, which might be due to the sampling year (June, 2014). *Hy. anatolicum* is a potential vector responsible for the transmission of *Theileria annulata* and *Theileria lestoquardi* in Pakistan (Ali et al., 2013; Durrani and Goyal, 2011). Furthermore, it can cause serious damage to cattle hides because of the long mouthparts. Notably, these ticks preferentially feed on the udder and teats of cattle (Bellew and Mekonnen, 2011; Mattioli et al., 1997; Tadesse et al., 2012) and may cause serious problems in the suckling of calves.

Rh. microplus was the second most prevalent tick species and also infested all the animal species in both the agro-ecological zones. A recent study from Punjab province also reported *Rh. microplus* as the second most common tick species in ruminants (Mustafa et al., 2014). Our finding that *Rh. microplus* was the dominant tick species in the semi-arid zone (Northern part of the province), is in accord with those of a local study conducted on two Government farms (Irshad et al., 2010), where the authors noticed a clear preponderance of *Rh. microplus*. The species was absent in the southern part of the province (MTN, BWP and RYK), which is a drier region. The geographical distribution and abundance of this tick species has been greatly promoted by the water retaining capacity of the underlying layer of the soil and the increased relative humidity (Raizada and Nagar, 1979). A lot of research work remains to be completed with previous records of *Rh. microplus* in Asia, as many of them have inadequate spatial references, and accession to the actual records to confirm the species determination is sometimes impossible (Estrada-Peña et al., 2006). Some reports indicated the high rainfall requirements of *Rh. microplus* in Africa (Estrada-Peña et al., 2006), and the species was absent in areas where rainfall was less (de Vos, 1979).

The ecological niche for *Hy. dromedarii* was confined to the Bahawalpur district in the arid zone from where it has already been reported, but always in small numbers (Hussain and Kumar, 1985; Siddiqi and Jan, 1986). The major part of this district consists of desert where the camel production is common and this tick species is specialized to feed on camels. Additionally, the confined presence of *Hy. dromedarii* to this district is perhaps explicable by the influence of low relative humidity on this desert-adapted tick species (Hussain and Kumar, 1985). Furthermore, the sequence of *Hy. dromedarii* showed 97% identity to a published sequence of *Hy. dromedarii* obtained from dromedary camel in India and this confirms our finding as Bahawalpur district shares its border with India.

Here, we report for the first time the occurrence of *Rh. turanicus* in Punjab province. Only limited numbers were found and the tick was confined to Bahawalpur and Rahim Yar Khan districts of the arid zone. The tick species was only found on water buffaloes and goats. Although a number of studies have been conducted in the past, *Rh. turanicus* has never been reported from Punjab province; however, it has been reported from Sindh province, but quite a long time ago (Hussain and Kumar, 1985). McCarthy (1967) also found *Rh. turanicus* in Pakistan, but he considered it as a subspecies of *Rh. sanguineus* due to the previous work of Pervomaisky (1954). Nevertheless, it has been identified on domestic ruminants in neighbouring countries, namely Iran (Abdigoudarzi

et al., 2011; Ganjali et al., 2014; Rahbari et al., 2007; Razmi et al., 2011), India (Chhillar et al., 2014; Ghosh et al., 2007), Bangladesh (Ghosh et al., 2007), China (Jing-Yun et al., 2015; Wang et al., 2015; Wei et al., 2015) and Iraq (Shubber et al., 2013). A report from Europe concluded that *Rhipicephalus sp. III* from Pakistan and India were morphologically and genetically close to, but still different from *Rh. turanicus* (Dantas-Torres et al., 2013). After considering all these reports, we conclude that *Rh. turanicus* has been there all the time, but was reported as *Rh. sanguineus*, as the two are morphologically very difficult to distinguish and molecular techniques were not utilized for confirmation.

These tick species infest a broad range of host species (Walker et al., 2003) and transmit several important pathogens including viruses, bacteria and protozoa of animals and human beings. *Rh. microplus* is considered the most important parasite of livestock in the world (Estrada-Peña et al., 2006). *Hy. anatolicum* has been reported as the principal tick vector for Crimean-Congo haemorrhagic fever (CCHF) (Mehlhorn, 2012; WHO, 2016). After several outbreaks of CCHF in Pakistan (Athar et al., 2005; Jamil et al., 2005), the infection has now become an endemic problem (Alam et al., 2013) and the possibility of transmission of CCHF virus to farmers, especially when hand picking is a very common practice, cannot be neglected (Muhammad et al., 2008). *Rh. microplus* and *Hyalomma* spp. are suspected to be potential vectors in the transmission of *A. centrale* and *A. marginale* (Jabbar et al., 2015), which is the cause of bovine anaplasmosis in Pakistan (Ashraf et al., 2013). Furthermore, these species have been reported to transmit different species of *Theileria* and *Babesia*, particularly highly pathogenic *T. annulata* and *B. bovis* in buffalo (*Bubalus bubalis*) and cattle (*Bos taurus indicus* and *Bos taurus taurus*) in Pakistan (Ali et al., 2013; Durrani et al., 2008; Jabbar et al., 2015). Furthermore, the zoonotic importance of *B. bovis* cannot be ruled out (Homer et al., 2000). Although scanty, the presence of *Rh. turanicus* is still of medical as well as veterinary importance as it is known to transmit *Coxiella burnetii*, causative agent of Q fever, which has recently been recorded in Pakistan (Shabbir et al., 2016; Zahid et al., 2016).

Besides the aforementioned tick species, the following species of the identified genera have also been reported from Pakistan; *Hyalomma* spp.: *Hy. aegyptium*, *Hy. excavatum*, *Hy. detritum*, *Hy. turanicum*, *Rhipicephalus* spp: *Rh. annulatus*, *Rh. sanguineus*, *Rh. appendiculatus* (Ghosh et al., 2007).

5.5 Tick species in animal species

Animals were infested with a single as well as multiple tick species, which has been reported by previous researchers (Anderson et al., 2013; Ghosh et al., 2007; Sajid et al., 2008). However, variation in the prevalences between single and mixed infestation exist, which can be attributed to the exposure of the animals and/or innate resistance to some species of ticks (Daynes and Gutierrez, 1980). *Hy. anatolicum* and *Rh. microplus* infested all ruminant species; *Hy. dromedarii* was absent in sheep, while *Rh. turanicus* was found only in buffaloes and goats. *Hy. dromedarii* and *Rh. turanicus* are generally more adapted to camels and small ruminants, respectively, as compared to bovines, and this might explain the small number of specimens collected in our study.

5.6 Tick-borne pathogens

In addition to the direct harms to domestic animals, ticks transmit a wider variety of infectious agents to animals and humans than any other blood-feeding arthropod (Munderloh et al., 2005). The pathogens they transmit include viruses, bacteria, fungi and protozoa (de La Fuente et al., 2015). The annual global economic loss due to ticks and tick-borne pathogens in cattle was estimated to amount to billions of US dollars (between 13.9 billion and 18.7 billion US$) (de Castro, 1997; Jongejan and Uilenberg, 2004). Although a number of TBDs (more than 16) of humans and animals (around 19) have been described in previous studies (Sonenshine and Roe, 2014) during last few years, the spectrum of TBDs has amplified and various TBDs, such as borreliosis, rickettsiosis, ehrlichiosis and anaplasmosis, acquire more attention from medical and veterinary clinicians (Dantas-Torres et al., 2012). Recently developed molecular biological tools lead to the discovery of numerous new pathogens (Dantas-Torres et al., 2012; Doudier et al., 2010; Ehounoud et al., 2016) and recent molecular diagnostic techniques, such as PCR and RLB hybridisation assay are powerful methods for characterising species and parasite polymorphisms, which facilitate describing population genetics and generating consistent epidemiological data (OIE, 2014b).

In our study, a total of 405 (*Hy. anatolicum* = 300, *Rh. microplus* = 89, *Hy. dromerdarii* = 9, *Rh. turanicus* = 7) tick pools (semi-arid zone = 113, arid zone = 292) were screened by RLB assay for the presence of DNA from 41 tick-borne pathogens belonging to *Anaplasma*, *Ehrlichia, Babesia, Theileria* and *Rickettsia* species. The RLB primers for *Anaplasma/Ehrlichia* and *Babesia/Theileria* target the V1 hyper-variable region of the 16S rRNA gene and V4 hyper-variable region of 18S rRNA gene, respectively, and to date have been found to be

conserved in all members of these genera (Bekker et al., 2002; Gubbels et al., 1999). The oligonucleotide probes used in the RLB assay were designed to be species-specific and to detect all members of a species, based on the assumption that the 16S and 18S hyper-variable regions are conserved within *Anaplasma/Ehrlichia* and *Babesia/Theileria* spp., respectively. Moreover, it is also useful to detect novel species if only a catch-all probe (either *Anaplasma/Ehrlichia* or *Babesia/Theileria*) gives a positive signal without a species-specific signal (Bekker et al., 2002; Chaisi et al., 2014, 2013; Mans et al., 2015; Oosthuizen et al., 2009). The RLB assay was shown to be a valuable tool for the simultaneous detection of several tick-borne pathogens and it can detect even low parasitemias (Aktas et al., 2011; Altay et al., 2008, 2007; Bekker et al., 2002). Additionally, the findings were confirmed by sequencing of 35 selected positive samples. Only one study from Pakistan has yet confirmed the identified tick-borne pathogen, i.e. *T. annulata*, through sequencing analysis (M. K. Khan et al., 2013).

Out of a total of 405 tick pools, DNA from one or more tick-borne pathogens was found in 148 (36.5%) pools. Among the positive pools, 94 (63.5%) had a mixed infection with two or more (ranging from 2 to 5) tick-borne pathogen species with 18 different combinations, whereas 54 (36.5%) pools were infected with single tick-borne pathogen species. The most common combination was of *Ehrlichia* sp. (Multan), *Ehrlichia* sp. ERm58 and *Ehrlichia* sp. Firat. However, the co-infection patterns of the identified tick-borne pathogens remains to be investigated, as each sample in the current study contained DNA from multiple (3 to 5) ticks, but of the same species. The overall prevalence estimates of tick-borne pathogens in Punjab province indicated that the prevalence of *Ehrlichia* spp. (22.2%) was highest, followed by *Theileria* (9.9%), *Anaplasma* (7.7%), *Babesia* (2.5%) and *Rickettsia* spp. (1%). We found that the prevalence of *Anaplasma* spp. in the semi-arid zone (15%) was significantly higher than in the arid zone (4.8%). The higher prevalence of *Rh. microplus* in the semi-arid zone and more common stall feeding practices could have possibly accounted for the higher prevalence of *Anaplasma* spp. In both the agro-ecological zones, *Babesia* spp. were the least prevalent tick-borne pathogen.

The study area is endemic for TBDs, both in buffaloes and cattle (Durrani and Kamal, 2008) and several studies have been carried out to understand the epidemiology of TBDs in various regions of Pakistan, but none of them, except (Robertson et al., 1970), attempted to screen the samples (neither blood nor ticks) for *Ehrlichia* and/or *Rickettsia* species. So far, only two studies have evaluated tick samples, but these researchers screened the ticks only for *T. annulata, T. lestoquardi*

and *T. ovis* (Ali et al., 2013; Durrani and Goyal, 2011), while the role of other tick species in the transmission of tick-borne pathogens in Pakistan have so far not been investigated (Jabbar et al., 2015). Most scientists in Pakistan relied on blood smear examination for the diagnosis of TBDs in their studies. Similarly, the identification of tick-borne pathogens and their genotypes was based on morphological examination using the blood smear method (Jabbar et al., 2015), which is often less reliable and not recommended by the World Organisation for Animal Health (OIE), at least not to estimate the prevalence of infection in animal populations and to contribute to policy development (OIE, 2014b). However, advanced molecular techniques like PCR and RLB assay are more specific and sensitive and can differentiate multiple pathogens simultaneously (Schnittger et al., 2004). Therefore, we utilized such molecular methods for the detection of ticks and tick-borne pathogens to produce more reliable data for future investigations. Moreover, many of the previous studies were limited to small areas without consideration of agro-ecological zones, production systems and sampling strategies; all of which can affect the study results (Jabbar et al., 2015).

5.6.1 Anaplasmosis

We identified four *Anaplasma* species, namely: *A. marginale*, *A. centrale*, *A. ovis* and *Anaplasma* sp. BL099-6 in four tick species from two agro-ecological zones. So far, a number of studies have reported *A. marginale* and *A. centrale* in cattle and buffaloes with a range of prevalences, i.e. 4 to 60%, which falls within the range for endemic areas, from all over the country (Afridi et al., 2005; Ashraf et al., 2013; Atif et al., 2012; Bhutto et al., 2012; Rajput et al., 2005; Sajid et al., 2014). We estimated that the prevalence of *A. marginale* was higher than the other *Anaplasma* species. The present study includes the first molecular detection of *A. marginale* in *Rh. microplus* and *Hy. anatolicum* ticks from Pakistan. However, the pathogen has been previously detected in bovine blood samples from Pakistan using blood smear examination, PCR-restriction fragment length polymorphism (RFLP)-based analysis and serological method, i.e. complement-enzyme linked immuno sorbent assay (cELISA) (Ashraf et al., 2013; Atif et al., 2013). Although the pathogen has been detected in *Rh. microplus* ticks in other parts of the world (Ehounoud et al., 2016; Pesquera et al., 2015; Ybañez et al., 2013), to the best of our knowledge, it has never been isolated from *Hy. anatolicum* ticks. Nevertheless, the potential of this tick species to transmit *A. margniale* needs further investigation, as pathogen DNA might have been present in the host's blood ingested by the tick. A recent article from Pakistan described that the *Hyalomma* and *Rhipicephalus* ticks could

be the potential vectors in the transmission of *Anaplasma* species in Pakistan; however, no evidence is available on experimental transmission of the pathogen (Jabbar et al., 2015). *A. marginale* has a worldwide distribution and is thought to be as one of the most prevalent tick-borne pathogens causing high morbidity and mortality in buffaloes and cattle in subtropical and tropical areas (Kocan et al., 2010). It causes bovine anaplasmosis which is one of the most important TBDs of ruminants worldwide (Zivkovic et al., 2007). The disease is characterised by fever, anaemia, jaundice, loss of appetite, decreased milk production, abortion in pregnant animals, and death, mainly in exotic animals (Camus and Uilenberg, 2010). Animals that recover from the disease remain in carrier state and can serve as reservoirs for mechanical and/or biological transmission (Camus and Uilenberg, 2010; McGuire et al., 1991). The pathogen is responsible for the majority of clinical cases all around the world, including Pakistan (OIE, 2015; Sajid et al., 2014). In our study, a significantly higher prevalence of *A. marginale* in the semi-arid zone than in the arid zone could be linked with the higher prevalence of *Rh. microplus*, which is primarily responsible for the transmission of this pathogen (Futse et al., 2003; Lew-Tabor, 2015). *A. centrale* is a naturally attenuated strain that has been employed as a live vaccine to prevent severe diseases due to *A. marginale* for 100 years (Herndon et al., 2010).

Our results showed that DNA of *A. ovis* was present in 6 (1.5%) tick pools. Hitherto, only one study has recorded *A. ovis* in small ruminants from KPK province (Talat et al., 2005) and there is a paucity of information on the epidemiology of ovine anaplasmosis in Pakistan. Although, *A. ovis* infections have been molecularly confirmed in other parts of the world (Aktas, 2014; Jalali et al., 2013; Noaman, 2012), molecular or serologic evidence to support *A. ovis* infection in Pakistani sheep or goats has never been reported. As the name indicates, *A. ovis* causes mild diseases in sheep and goat, particularly if the animals are in stress, but the infections may sometimes produce severe clinical disease, which can even lead to death (Aktas et al., 2009; Friedhoff, 1997; Stoltsz, 2004). In our study, *A. ovis* was found in *Hy. anatolicum*, *Hy. dromedarii* and *Rh. microplus* ticks. Similarly, *Hy. anatolicum* has been recently shown as one of the important vectors responsible for the transmission of ovine anaplasmosis in Iran (Jalali et al., 2013; Noaman, 2012). However, the vectors of this pathogen are not clearly known in many areas of the world (Aktas et al., 2009; Friedhoff, 1997).

Here, we document the first evidence of the presence of *Anaplasma* sp. BL099-6 in Pakistani ticks, as two sequences of *Anaplasma* species (from *Hy. anatolicum* and *Rh. turanicus*) showed 98%

identity to the uncharacterised *Anaplasma* sp. BL099-6 isolated from *Hyalomma asiaticum* in Xinjiang, China (Kang et al., 2014). Although *Anaplasma* sp. BL099-6 infections have been reported from other parts of the world (Kang et al., 2014), little is known about the pathogenicity, vectors, and reservoir host of *Anaplasma* sp. BL099-6 and further epidemiological studies should be performed. The reason for the identification of this new *Anaplasma* species could be that almost all previous studies, except the two where the authors utilized PCR-RFLP and cELISA, were based on conventional method, i.e. stained blood smear, which is very subjective and not very helpful to differentiate on species level. Other important limitations of these studies were that they were conducted in the peripheries of important veterinary research institutions or close to public livestock research stations, indicating a sampling bias. In addition, most of the studies relied on convenient sampling technique that could be another potential source of bias (Jabbar et al., 2015).

5.6.2 Ehrlichiosis

In our study, five *Ehrlichia* species, namely: *Ehrlichia* sp. (Multan), *Ehrlichia* sp. ERm58, *Ehrlichia* sp. Firat, *Ehrlichia* sp. Omatjenne and *E. mineirensis* were identified in three tick species. The former four species are still uncharacterised and *E. mineirensis* (previously known as *Ehrlichia* sp. UMFG-EV) has been recently characterised after its isolation from *Rh. microplus* from Minas Gerais, Brazil (Cabezas-Cruz et al., 2015). *Ehrlichia* species are responsible for emerging and re-emerging tick-borne zoonoses that cause life-threatening diseases in domestic animals (Cabezas-Cruz et al., 2015). As far as we know, this is the first study that illustrates the presence of *Ehrlichia* species in ticks from Pakistan. Recently, a case report indicated that *Ehrlichia canis* occurred as a co-infection with *Babesia gibsoni* in a canine blood sample, however, the authors relied only on the blood smear examination and had not utilized any molecular technique for further confirmation of the organism (Abbas et al., 2015). Since there is neither a clinical nor a laboratory-based diagnostic and disease surveillance system in Pakistan pertaining to ehrlichiosis, the disease usually remains unreported. Nevertheless, various *Ehrlichia* species have been identified in tick samples from the border countries: China (Wen et al., 2003, 2002) and India (Abd Rani et al., 2011; Das and Konar, 2013).

Among the identified *Ehrlichia* species, the most common sequence found was that of another hitherto uncharacterised species, i.e. *Ehrlichia* sp. (Multan) (18.0%), with 99% identity to sequences of the 16S rRNA gene of *Ehrlichia* sp. Firat 3 from Eastern Turkey (Aktas et al., 2009) and *Ehrlichia* sp. BL157-4 from Xinjiang, China (Kang et al., 2014). Notably, the hypervariable

region 16S rRNA is well conserved within the same species (Cruz et al., 2012) and diverse between *Ehrlichia* species (Warner and Dawson, 1996; Wen et al., 2002). Although very small variations in the 16S rRNA gene sequence might be due to PCR inaccuracies or sequencing errors, these variations might also be due to differences in the sequences of strains of the same species (Aktas et al., 2009). Nevertheless, new *Ehrlichia* species continue to be discovered with the recent characterisation of a novel *Ehrlichia* species, which was first found in blood samples of Canadian cattle (Gajadhar et al., 2010) and later in the haemolymph of *Rh. microplus* ticks in Brazil (Aguiar et al., 2014; Cruz et al., 2012). Additionally, many other potential novel candidates have been detected, e.g. *Ehrlichia* sp. HF565, *Ehrlichia* sp. 360, *Ehrlichia* sp. Tibet and *Ehrlichia* sp. Fujian, all identified in ixodid ticks and mainly characterised by sequencing analysis (Telford Iii et al., 2011; Cruz et al., 2012). We found that DNA from *Ehrlichia* sp. (Multan) was present in *Hy. anatolicum* (62 tick pools), *Rh. microplus* (10) and *Hy. dromedarii* (1), however, transmission competence for these tick species requires remains to be demonstrated. Furthermore, this new *Ehrlichia* genotype was extensively distributed in both the agro-ecological zones. The preliminary sequencing results obtained for the *Ehrlichia* species suggest the presence of a potential novel *Ehrlichia* species in Pakistan. However, additional studies are required to confirm whether this new genotype corresponds to a new *Ehrlichia* species or if it is a strain of a previously characterised species.

We found that *Ehrlichia* sp. ERm58 was the second most common *Ehrlichia* species followed by *Ehrlichia* sp. Firat. *Ehrlichia* sp. ERm58 was first time detected in *Rh. muhsamae* recovered from cattle in Mali, Africa (Parola et al., 2001) and since then new *Ehrlichia* genotypes, e.g., (*Ehrlichia* sp. EBm52, *Ehrlichia* sp. BL157-9, *Ehrlichia* sp. BL157-6 and *Ehrlichia* sp. BL157-4) closely related to this have been detected (Kang et al., 2014; Parola et al., 2003). *Ehrlichia* sp. Firat was initially isolated from *Hy. anatolicum* ticks collected from an animal shelter in Turkey (Aktas et al., 2009). However, little is known about these *Ehrlichia* species and further studies are required to detect natural infections in vertebrates and potential involvement in the causation of disease in humans or animals.

The majority of the sequences of *Ehrlichia* species were divergent from any known *Ehrlichia* species and only four of the sequences shared 89 to 99% identity with *E. mineirensis*, recovered from *Rh. microplus* from Minas Gerais, Brazil (Cabezas-Cruz et al., 2015). However, contrary to the findings of the latter report, we could not detect *E. mineirensis* in *Rh. microplus* ticks and all

the samples (13, 3.2%) positive for *E. mineirensis* were from to *Hy. anatolicum* ticks (Aguiar et al., 2014). Similarly, *Ehrlichia* sp. Omatjenne was only present in *Hy. anatolicum* ticks from the arid zone. This *Ehrlichia* species was first time isolated from *Hy. truncatum* tick from Namibia (Allsopp et al., 1997). Recently, it was reported in blood samples from naturally infected buffalo (Eygelaar et al., 2015) and cattle (Aktas and Özübek, 2015; Mtshali et al., 2013). Although cattle have been suggested as a natural host for this ehrlichial agent (Aktas and Özübek, 2015), there is limited information available on vectors and reservoirs for this species. Our results reveal that *Hy. anatolicum* ticks might be a potential vector responsible for the transmission of *E. mineirensis* and *Ehrlichia* sp. Omatjenne in Pakistan, however, this needs to be demonstrated through experimental studies.

This is the first report from Pakistan confirming the presence of multiple *Ehrlichia* species and their potential vectors, hence, it will provide a better insight into the epidemiology of ehrlichiosis in this region. Since the majority of the known *Ehrlichia* species cause human zoonoses, it is suggested that further studies should be carried out to know about their vector-competence of various tick species, the pathogenicity of the detected *Ehrlichia* species and potential implications to human or animal health. In addition, human and veterinary clinicians should consider ehrlichiosis among the differential diagnoses when TBDs are suspected.

5.6.3 Rickettsiosis

Rickettsiosis is caused by *Rickettsia* spp. belonging to the spotted fever group (SFG) that infects humans, domestic animals and wildlife (Lopez-Velez et al., 2015; Maina et al., 2014; Wei et al., 2015). These are newly emerging vector-borne infections distributed worldwide (Harrus et al., 2011; Parola et al., 2005). In the present study, rickettsial DNA was found in four tick pools, of which two were positive for *R. massiliae,* one for *R. raoultii* and one for both the *Rickettsia* species. As this is the first report that describes the presence of *R. massiliae* and *R. raoultii* in Pakistan, we confirmed our findings by sequencing. *R. raoultii* has recently been detected and characterized by Mediannikov and his co-workers (Mediannikov et al., 2008). Although the progression of knowledge on rickettsiosis in the Indo-Pakistan has an extended and complex history, to date, information on rickettsial agents in this huge territory remains inadequate. As far as we know, the only study, which described rickettsiosis in Pakistan, was carried out between 1963 and 1965 in West Pakistan (Robertson et al., 1970). These authors examined six tick species that were collected from various animal species, including domestic and wild animals, and isolated three *Rickettsia*

species: *Rickettsia conorii* (cause of Indian tick typhus also known as Mediterranean spotted fever), *R. sibirica* (cause of North Asian tick typhus also called Siberian tick typhus) and an unknown *Rickettsia* species that was serologically distinct from other known SFG *Rickettsiae* (Robertson and Wisseman, 1973). Interestingly, these authors found all five strains of novel *Rickettsia* species (JC880, JD 112, JC494, JC687, JC685§) only in *Rhipicephalus* ticks. Moreover, the specimens of this potentially new *Rickettsia* species were isolated from Lahore and Sialkot districts, which are not far from Attock and Okara districts, where we found *R. massiliae* or *R. raoultii*. These findings support our results, as these authors might have found *R. massiliae* or *R. raoultii* in tick samples, but they could not confirm, because these species remained unknown during that period. Although the authors suggested further studies for the confirmation of this new *Rickettsia* species, to date, no study has considered these novel findings (Robertson and Wisseman, 1973). More surprisingly, since then results of studies investigating the presence of *Rickettsia* species in blood or ticks in the region have not been published. Therefore, we re-confirmed the presence of *Rickettsia* species in Pakistan. However, *R. massiliae* and *R. raoultii* have recently been reported from a neighbour country (China) (Guo et al., 2015; Kang et al., 2014; Tian et al., 2012). These pathogens have never been described in South Asia, which includes Afghanistan, India, Pakistan, Bangladesh, Sri Lanka, Nepal, Bhutan and the Maldives. These *Rickettsia* species are important members of SFG group. Given that these pathogens were isolated from ticks recovered from domestic animals, it would seem to have ample chances of human infection, particularly, when farmers live in a close association with animals, which is a quite common husbandry practice in Pakistan, especially in the study area, where people live with animals under the same roof. Furthermore, weather conditions in most parts of Punjab province are very warm in summer, which may lead to more tick bites and hence result in increased pathogen transmission (Parola et al., 2008). Nevertheless, with the lack of appropriate diagnostic facilities in developing countries like Pakistan, rickettsiosis on most instances remains mis- or undiagnosed, and tends to be grouped with other diseases exhibiting a similar disease outcome such as fever of unknown origin. Rickettsial infections, particularly mis- and underdiagnosed, are important public health concerns, as they might put considerable financial burden on poor families (Rathi and Rathi, 2010). Therefore, it is suggested that infections with these pathogens should be considered when tick-borne diseases are suspected.

R. massiliae was first described in *Rh. sanguineus* ticks collected near Marseille in 1992 (Beati and Raoult, 1993), and since then it has been described in numerous *Rhipicephalus* species in Greece (Babalis et al., 1994), Central African Republic (Dupont et al., 1994), Portugal (Bacellar et al., 1995), Spain (Beati et al., 1996), Mali (Parola et al., 2001), Switzerland (Bernasconi et al., 2002), Sardinia (Italy) (Mum et al., 2008), Ivory Coast (Berrelha et al., 2009), Senegal (Mediannikov et al., 2010), Guinea (Mediannikov et al., 2012), Nigeria (Reye et al., 2012), China (Wei et al., 2015) and Israel (Ereqat et al., 2016). In agreement with previous reports, tick pools infected with *R. massiliae* belonged to *Rhipicephalus* ticks except for one that belonged to *Hy. anatolicum* recovered from Goat in Okara district. However, other *Rickettsia* species such as *R. africae*, *R. barbariae* and *R. raoultii* have been isolated from *Hyalomma* ticks (Ereqat et al., 2016; Guo et al., 2015; Waner et al., 2014; Wen et al., 2014). After its first description in humans in 2005, *R. massiliae* infections have been reported in Europe and South America (García-García et al., 2010; Parola et al., 2013). Infections with *R. massiliae* are commonly distinguished by fever, night sweats, maculopapular rash and necrotic eschar (Cardeñosa et al., 2003; Cazorla et al., 2008; Ereqat et al., 2016; García-García et al., 2010). *R. massiliae* has natural resistance to rifampin, which means that the pathogen may cause a Mediterranean spotted fever-like disease, which has been reported from the Indo-Pakistan region (Parola et al., 2005; Rathi and Rathi, 2010; Vitale et al., 2006).

A novel finding in our study was the detection of *R. raoultii* DNA in *Rh. microplus* ticks recovered from cattle and buffalo from Attock and Okara districts, respectively. Nevertheless, based on the information in GenBank, *R. raoultii* has been identified in 14 tick species, namely: *Amblyomma helvolum, Dermacentor nuttallii, D. marginatus, D. reticulatus, D. silvarum, Haemaphysalis concinna, Ha. japonica, Hyalomma asiaticum*, *Hy. lusitanicum* and *H. erinacei*, *Ixodes persulcatus, I. ricinus*, *Rhipicephalus pumilio, Rh. turanicus* (Guo et al., 2015; Wen et al., 2014). However, the presence of *R. raoultii* DNA in *Rh. microplus* does not imply a transmission competence for this tick species, as the ticks might have been fed on infected animal but could still be unable to transmit the infection. To the best of our knowledge, this is the first report from Pakistan that will add significantly in understanding towards the distribution of *R. massiliae* and *R. raoultii*.

5.6.4 Babesiosis

The results of this survey indicated the presence of *B. bigemina*, *B. bovis*, *B. caballi*, and *B. occultans* in two tick species (*Hy. anatolicum* and *Rh. microplus*) from Punjab province. *B. bigemina* and *B. bovis* have previously been identified in bovine blood samples and antibodies against *B. caballi* have been detected in equine blood samples from Pakistan (Ahmad et al., 2014; Atif et al., 2012; Chaudhry et al., 2010; Hussain et al., 2014; Zulfiqar et al., 2012). *B. bovis* and *B. bigemina* are mainly responsible for causing animal babesiosis, which is considered one of the highest ranked TBDs affecting more than a billion cattle globally (Figueroa et al., 2010). The main impact of animal babesiosis is on dairy industry, however, infections occur also in other animal species, including goats, sheep, dogs, horses and pigs (Carter and Rolls, 2015; Chaudhry et al., 2010). The disease can cause high mortality (up to 50%) in affected herds (Antoniassi et al., 2009; El-Ashker et al., 2015). *B. bovis* is more pathogenic than the other species and infections with this pathogen are characterised by high fever, anorexia, abortion in pregnant animals, extensive erythrocytic lysis resulting in anaemia, jaundice and haemoglobinuria, neurological symptoms (rarely), and death in severe cases (Bram, 2016; Figueroa et al., 2010). However, the outcome of the disease depends on host-related factors, such as age, breed, and immune status (Figueroa et al., 2010). Comparatively, *B. bigemina* infections produce less severe symptoms, which may at least partially resemble those of *B. divergens* infections (OIE, 2010; Zintl et al., 2003). *B. bovis* is prevalent in Asia, Africa, Australia, Central and South America and Europe, whereas *B. bigemina* has been reported from Asia, Africa, Europe and the Far East (Bram, 2016). *B. bovis* and *B. bigemina* were only present in the semi-arid zone. This could be attributed to the clear preponderance of *Rh. microplus* ticks, which is a principal vector for the transmission of these parasites (Figueroa et al., 2010; OIE, 2010). Although *Rh. microplus*, is very common in Pakistan, hitherto, none of the previously conducted studies assessed the role of this tick species in the transmission of tick-borne pathogens. Here, for the first time, we screened *Rh. microplus* ticks from Pakistan for the presence of various tick-borne pathogens.

Although, previous reports from Pakistan confirmed the endemicity of babesiosis in different livestock species with variation of prevalence estimates within different parts of the country, this is the first report that describes the occurrence of *B. occultans* in Pakistan, thus extending the distribution of this species to South Asia region that will provide a greater insight into epidemiology of *Babesia* species. According to the sequence analysis, the Pakistani *B. occultans*

sequence showed 99% identity to the *Babesia occultans* isolate HD-127 recovered from *Rh. sanguineus* from Gansu, China (Yu et al., 2016). *Babesia* occultans was first time recovered from *Hyalomma marginatum rufipes* in South Africa in 1981 (Gray and De Vos, 1981). Afterwards, for a long time, the geographical distribution of this species was thought to be restricted to sub-Saharan African countries (Dipeolu and Amoo, 1984; Ros-García et al., 2011), but recently the species has been identified in cattle blood and *Hyalomma* ticks from Tunisia - northern Africa (Ros-García et al., 2011), the Balearic Islands, Spain (Ros-García et al., 2012), southern part of Italy (Decaro et al., 2013) and Turkey (Aktas and Ozubek, 2015). Additionally, the parasite has been recently found in blood samples collected from dogs in India, a neighbour country (Mandal et al., 2014). Albeit, *B. occultans* has been found in ticks as well as cattle blood, it has never been associated with the clinical disease in animals and even experimental infections in splenectomized animals proved apathogenic (Gray and De Vos, 1981; Ros-García et al., 2011). However, a recent report from the southern part of Italy associated the occurrence of *B. occultans* with the clinical outbreak of bovine piroplasmosis (Decaro et al., 2013). The authors observed pale mucosae, pyrexia and loss of milk production, however neither gastroenteric nor respiratory signs were recorded (Decaro et al., 2013). Future studies are required to explore the pathogenicity and to get a better picture of geographical distribution of this species. *B. occultans* and *B. caballi* were prevalent only in the arid zone which might be due to the increased abundance of *Hyalomma* ticks, an important vector for these pathogens (Aktas et al., 2014; Orkun et al., 2014; Ros-García et al., 2012, 2011).

5.6.5 Theileriosis

The most studied bovine TBD in Pakistan is theileriosis. In our study, three species of *Theileria* (*T. annulata*, *T. orientalis*, and *T. ovis*) were recovered from two tick species (*Hy. anatolicum* and *Rh. microplus*) from Punjab province. The identified pathogens were anticipated, except *T. orientalis*, as they have been previously reported in animal and/or tick species from Pakistan (Ali et al., 2013; Durrani and Goyal, 2011; Durrani et al., 2012; Khattak et al., 2012; Shahzad et al., 2013).

We found that *T. annulata* sequences were 100% identical to *Theileria annulata* clone Zhangye 27 (a recently submitted sequence) detected in Bactrian camel blood from China. A previous study from Pakistan reported that some of the *T. annulata* sequences were identical to those from Turkey, while others were novel, suggesting a genetic distinctiveness (M. K. Khan et al., 2013). A recent

study from a bordering country also found new genotype of *T. annulata* (George et al., 2015). However, *T. annulata* sequences in the former study were from Okara and Sheikhupura districts, but we could not find any *Theileria* species in Okara district and Sheikhupura district was not included in the sampling. The prevalence of *T. annulata*, which causes tropical theileriosis, was highest (6.7%), followed by *T. orientalis* (3.5%), and *T. ovis* (0.2%). Among these species, *T. annulata* is the most virulent and has numerous strains which are broadly distributed in different geographical regions of the world. *T. annulata* produces a severe and potentially fatal disease in cattle, resulting in substantial economic losses in the dairy industry in Africa and Asia (Bishop et al., 2009; Jabbar et al., 2015). The disease is more acute in exotic and cross-bred cattle, where the case-fatality rate can reach up to 80%, as compared to indigenous cows, where the case-fatality rate is usually around 20% (Jabbar et al., 2015; Ouhelli, 1991). Although a number of *Hyalomma* spp. are known to transmit *T. annulata*, a single study has so far detected *T. annulata* in *Hy. anatolicum* and *Hy. dromedarii* ticks removed from cattle in Pakistan (Ali et al., 2013). However, these authors could not find *T. annulata* in *Hy. marginatum*, *Boophilus annulatus* and *Amblyomma variegatum* ticks. Similarly, we found that *T. annulata* was mainly present (96.2%, 26/27) in *Hy. anatolicum* ticks and only one tick pool comprising of *Rh. microplus* ticks contained *T. annulata* DNA. Another study from Pakistan identified *T. lestoquardi* and *T. ovis* in *Hy. anatolicum* and *Rhipicephalus* ticks, respectively (Durrani and Goyal, 2011). These findings might suggest that *Hyalomma* spp. are mainly responsible for spreading *Theileria* infections in the livestock population in Pakistan.

This study represents the first evidence of the presence of *T. orientalis* in Pakistan. Although *T. orientalis* infections have been documented in cattle, African buffalo, water buffalo and Yak from all the major continents of the world (Chaisi et al., 2013; Sivakumar et al., 2014), including neighbouring countries of Pakistan, e.g. India (Aparna et al., 2011; Kakati et al., 2015) and Sri Lanka (Sivakumar et al., 2012), no study has so far reported *T. orientalis* (also known as *T. buffeli*) in Pakistan (Jabbar et al., 2015). Both names, *T. orientalis* and *T. buffeli*, continue to be used in the literature depending on the historical background of different scientific groups (Chaisi et al., 2014; Mans et al., 2015; Sivakumar et al., 2014), but the name *T. orientalis* is now widely accepted and used for the genotypes present in Asia, Australia and New Zealand (Hammer et al., 2015). The parasite has been considered for a long time as benign (Cufos et al., 2012; Kamau et al., 2011; Kamio et al., 1990), but pathogenic genotypes, mainly *ikeda* and *chitose* in the Asia-Pacific region

(Izzo et al. 2010; Kamau et al. 2011; Perera et al. 2014) have been found in many countries including China (Liu et al., 2011), India (Aparna et al., 2011), Korea (Baek et al., 2003), Japan (Yokoyama et al., 2012), Australia (Eamens et al., 2013; Islam et al., 2011) and New Zealand (McFadden et al., 2011). Clinical manifestations of oriental theileriosis usually include pyrexia, anaemia, jaundice, lethargy, weakness, abortion in female animals and mortality (Aparna et al., 2011; Islam et al., 2011; Izzo et al., 2010; McFadden et al., 2011). It is unclear how *T. orientalis* was introduced into Pakistan, but it may be speculated that this could have occurred by the importation of cattle from the State of Victoria in Australia (Jabbar et al., 2015), where the pathogen is now endemic (Perera et al., 2014; Piyumali K Perera et al., 2015; Piyumali K. Perera et al., 2015). Thousands of dairy cattle are imported to Pakistan and blood samples from these animals are not screened using molecular assays for the presence of piroplasms prior to export (Jabbar et al., 2015). Moreover, it has previously been suggested that the prevalence and intensity of infection of *T. orientalis* in cattle should be estimated upon arrival to Pakistan (Jabbar et al., 2015). In addition to that, there is a considerable illegal live animal transport between Pakistan and India (especially through the border with Rajasthan), where it has been previously reported (Appleby et al., 2008; Kakati et al., 2015). The accidental importation of ticks on exotic animals during the international trade of live animals has also played an important role for the spread of tick species and TBDs (de La Fuente et al., 2015).

We suggest that further studies should be carried out to estimate the prevalence of *T. orientalis* genotypes in cattle, water buffaloes and wildlife in Pakistan. *T. orientalis* is mainly transmitted by *Haemaphysalis* ticks that have been recovered from bovines in Pakistan (Durrani and Kamal, 2008). However, the pathogen has been detected in *Rh. microplus* from India (Kakati et al., 2015) and Vietnam (Khukhuu et al., 2011) and *Dermacentor nuttalli* in Mongolia (Altangerel et al., 2011). Thus, this is the first report of the involvement of *Hy. anatolicum* as a possible vector of *T. orientalis*, but experimental studies are required to confirm the role of this tick species in the epidemiology of *T. orientalis*.

Contrary to the findings of previous studies (Durrani et al., 2011; F. Iqbal et al., 2013; Saeed et al., 2015), we could not detect *T. lestoquardi* in our samples, even though the main proven vector (Razmi et al., 2003; Uilenberg, 1997), *Hy. anatolicum*, was widely distributed. However, these authors utilized a simple PCR technique and did not confirm their findings through further molecular analysis, i.e. sequencing.

5.7 Risk factors associated with higher tick prevalence on livestock farms

In addition to univariable statistical analysis, we used multivariable logistic regression model to identify the risk factors associated with high tick prevalence on livestock farms. Multivariable analysis has many advantages over a univariable analysis such as control for numerous confounders. It gives also more insight into potential relationships and interactions among variables and provides odds ratio (OR) adjusted for other variables, including confounders (Pourhoseingholi et al., 2012).

We assessed the effect of various determinants on tick prevalence. Our results showed that traditional rural housing was positively associated with higher tick prevalence and the odds of acquiring higher tick prevalence on farms with traditional housing type was as high as 13 times than farms with open housing system. In traditional rural housing a farm has covered and uncovered area, but without any specified proportion. The covered area consists of completely closed room/s without proper ventilation and a simple roof structure called "chappar". The rooms are used for protection from cold weather during the winter season, while the roof structure along with trees is used for protection during the summer and the monsoon season. The walls of the buildings are made of hard or soft bricks with mud as a seal, whereas the roof is made of bricks placed on wood or iron rods with a thin layer of mud on top.

A previous study has shown that the animals were more prone to tick infestation in a closed-type of housing, which is quite similar to the traditional housing system, as compared to open-type housing (A. Iqbal et al., 2013). It is hypothesized that less exposure to sunlight favors the retention of humidity in heaps of dung cakes and stacks of bricks in the closed houses, providing more and better breeding places for ticks. In addition, female ticks generally lay eggs in cracks and crevices in the walls of animal sheds, which provides a favorable environment for tick growth and development (Muhammad et al., 2008). Crevices in the walls are also a preferred hiding spot for *Hyalomma* nymphs and adults. Moreover, closed farms create favorable sheltering places for egg laying and hatching of ticks throughout the year (Jouda et al., 2004). Caulking of the walls ('teep' in Urdu') of the animal sheds is an inexpensive measure that significantly reduces the tick burden (Muhammad et al., 2008).

We found that the farms where grazing was practiced had a higher tick prevalence as compared to farms with stall feeding system. The odds of getting a high tick prevalence on farms where grazing

had been practiced were almost 12 times as high as on farms where animals were stall-fed. This might have resulted in less exposure of routinely stall-fed animals to the tick infestation from widespread-infested pastures (Ghosh et al., 2007). Similar findings have been recorded in a local study in Toba Tek Singh district, where the tick prevalence was quite low in stall-fed animals (A. Iqbal et al., 2013). Grazing has been reported as a risk factor for high tick prevalence in cattle in Bangladesh (Kabir et al., 2011). A negative association between zero-grazing and TBDs (babesiosis and theileriosis) have been recorded, most likely because confined animals were less exposed to ticks than outdoor grazing animals (Phiri et al., 2010). Animals that spent more time in grazing in the pasture lands were more heavily infested as compared to those that spent limited time in the field (Lorusso et al., 2013).

Another important risk factor associated with higher tick prevalence on farm was the absence of rural poultry. Conversly, the farms that reared rural poultry by integrated farming with ruminants had a significantly lower ($P = 0.006$) tick prevalence. The OR suggested that the chances of getting higher tick prevalence on farms, where rural poultry was absent, were 4.4 times as high as on farms where rural poultry was reared. Rearing chicken on livestock farms greatly reduced tick burden on the infested animals as the chicks picked ticks from the animal bodies as well as from their surroundings (Muhammad et al., 2008). Moreover, bovines that are tethered under trees in summer had a low tick burden due to predation of ticks by birds (Muhammad et al., 2008). On the one hand, integrated poultry and dairy farming is beneficial, but on the other hand, it is associated with considerable wastage of cattle feed and the risk of occurrence of infectious diseases like cryptococcosis and salmonellosis (Muhammad et al., 2008).

The use of acaricides was positively associated with low tick prevalence and the farms where acaricides were used had a low tick prevalence. Application of acaricides on farms has been reported the most widely used method of tick control in dairy farming (Muhammad et al., 2008).

Numerous plants have shown acaricidal, growth retarding and anti-molting activities. Many studies evaluated the efficacy of plant extracts on tick species. Initial findings of a study from India (Ghosh et al., 2007), which assessed the effect of alcoholic extracts of neem (Azadirachta indica) and sitaphal (Annona squamosa) against different developmental stages of *Hyalomma* and *Boophilus* ticks, showed promising results.

A few farmers also reported use of "Taramira", seeds of aragula (*Eruca sativa*), to control tick infestation. Although it may have somehow lowered tick burdens, it failed to decrease the tick prevalence significantly. In Punjab province, Taramira is used in different ways against ticks, e.g. as "Taramira oil" topically on the animal body, whereas others prepare a mixture with salt and water and drench tick-infested animals. This formula is not only believed to decrease tick burdens, but it is also considered as a milk booster (Muhammad et al., 2008).

In our study, flooring showed a statistically significant association ($P = 0.033$) with high tick infestation in univariable analysis and the farms with soft floors had a higher tick prevalence, but it appeared insignificant when included in the multivariable analysis, when confounding effects were accounted for. Previous studies from Pakistan reported non-cemented (soft and mixed type) flooring as a risk factor and the chances of being infested with ticks were 2 times as high as in animals kept on cemented floors (A. Iqbal et al., 2013).

Frequency of disposal of animal wastes (dung) was also significantly associated ($P = 0.031$) with high tick infestation in univariable analysis and the farms that used to dispose of animal dung on daily basis had low tick prevalence as compared to those where dung was removed on a monthly basis, but in multivariable analysis this factor was not statistically significant.

5.8 Attachment site preferences

Attachment site preferences of various tick species and their stages on a number of hosts, including domestic and wildlife species, have been studied. For instance, *Rh. sanguineus* on dogs (Dantas-Torres and Otranto, 2011), *Ix. rubicundus* on dairy cows (Fourie and Horak, 1993), *Ix. ricinus* on roe deer (Kiffner et al., 2011) and sheep (Ogden et al., 1998), *Hy. truncatum* and *Hy. marginatum* on sheep (Fourie and Kok, 1995), multiple tick species on goats and sheep (Vathsala et al., 2008), and *Rh. sanguineus* and *Ha. leachi* on dogs (Jacobs et al., 2001).

In our study, the distribution of tick species, their stages and sexes were noticeable different among host body areas. Attachment site preferences significantly varied by animal species for the tick species. Similarly, variations in the frequency of tick infestation on different body parts in buffaloes (Anderson et al., 2013; Kabir et al., 2011) and cattle (Asmaa et al., 2014) have previously been recorded. Immature ticks of *Hy. anatolicum* were present in all the four regions, whereas immature ticks of *Rh. microplus* were exclusively absent in the tail region in both animal species. In buffaloes, adults of both the tick species were found in increasing order of abundance in the ear,

brisket, udder/groin and tail regions. (Yakhchali and Hasanzadehzarza, 2004) found that the groin and mammary glands were the most infested regions in buffaloes. In cattle, the attachment density of *Hy. anatolicum* adults was highest in the udder/groin region as compared to *Rh. Microplus,* which was highest in brisket region. Similarly, another study reported that the axilla and udder/groin were the most common predilection sites in dairy cows (L'Hostis et al., 1994). A previous study from Pakistan also concluded that the attachment site preferences of *Hy. anatolicum* and *Rh. microplus* ticks were different from each other (Ahmed et al., 2012). In buffalo, the brisket region was least infested, whereas in cattle, the tail region harbored a quite low tick loads. This low number of ticks in these regions could be due to the fact that the skin in the brisket region of buffaloes is tight as compared to cattle which hinders in the attachment of ticks, whereas in cattle the tail region has very short and less hairs as compared to buffaloes, which is not an ideal condition for the attachment of ticks. Furthermore, a positive association between hair length and number of ticks has been reported (Veríssimo et al., 2002).

The male to female sex ratio of *Hy. anatolicum* was greater than 1, which is in agreement with previous reports from Pakistan (Ali et al., 2013), Asela (Tessema and Gashaw, 2010), Iran (Razmi et al., 2011), and Turkey (Aktas et al., 2004), in which the authors reported a male bias in their samples. Similar findings in case of other tick species, e.g. *Rh. evertsi evertsi*, *Am. variegatum*, *Am. cohaerens*, *Hy. truncatum* and *Hy. rufipes,* have been observed in previous studies (Gedilu et al., 2014; Huruma et al., 2015; Tadesse et al., 2012). This male bias could be explained by the reproductive behaviour of the female tick, as after a complete blood meal they drop off to the ground to lay eggs (Tadesse et al., 2012). In contrast, the male tend to stay on the host for a longer period to continue feeding and to mate (Horak et al., 2007). Another plausible explanation could be that the engorged females detached from the host more easily than males by host grooming behavior (Horak et al., 2006). In our study, female specimens of *Rh. microplus* outnumbered, possibly as a results of the small size of males, which may have been left unobserved during sampling (Tessema and Gashaw, 2010). Similar findings have been recorded in previous reports from Pakistan (Ali et al., 2013) and Turkey (Lorusso et al., 2013; Sayin et al., 2003).

5.9 Recommendations

1) Although acaricides were regularly used only on a few farms, ticks were found on all livestock farms, which might point to the occurrence of acaricidal resistance. Moreover, the existing methods used to control ticks and TBDs in Pakistan depends mainly on tick control using

acaricides, but, to date, no study has evaluated acaricidal resistance in ticks. Therefore, there is an urgent need to assess the acaricidal resistance in Pakistani ticks.

2) A potential novel *Ehrlichia* sp. was found in both the agro-ecological zones. Further studies are required to clarify whether these new genetic variants represent a new species, and if so, it should be further characterized, also at the molecular level, and its potential pathogenic role for animals and humans needs to be determined.

3) As the *Rickettsia* species, that are potential zoonotic agents and cause spotted fever, were described for the first time in Pakistan, clinicians should consider the possibility of spotted fever among the differential diagnoses. Moreover, in future research, *Rhipicephalus* ticks from wildlife, transported livestock, migrant birds, and human beings should be screened for the presence of rickettsial agents.

4) Crimean-Congo Hemorrhagic Fever (CCHF) virus is endemic in Pakistan and its principal vector, i.e. *Hyalomma* ticks, is distributed all over the country. Therefore, while removing ticks manually, consideration should be given to the potential hazard to humans due to the possible presence of CCHF virus in the ticks. More importantly, awareness programmes should be launched to inform farmers about the possibility of CCHF transmission by ticks. Moreover, ticks should be screened for the presence of CCHF virus.

5) Control of ticks and TBDs poses an unprecedented challenge for the farmers due to the suitability of environmental conditions for tick multiplication in the studied regions in Pakistan and the possible resistance of ticks to acaricides. Thus, integrated livestock farming with rural poultry and an open housing system should be promoted in small dairy holders.

6) As there is little information available on the epidemiology of ticks and tick-borne pathogens in the wildlife of Pakistan, future studies should also focus on wild animals to investigate whether they act as a reservoir for tick-borne pathogens that are transmissible to livestock.

7) Experimental studies to assess the role of *Hy. anatolicum* and *Rh. microplus* in the transmission *A. marginale* and *R. raoultii*, respectively, should be carried out.

6 Summary

Epidemiology of Ticks and Tick-borne Pathogens in the Semi-arid and the Arid Agro-ecological zones in Pakistan

Ticks and tick-borne diseases have a large impact on animal health and the livelihood of livestock owners, particularly in developing countries. Despite the suitability of Pakistan's climate for ticks, there is a paucity of systematic work investigating these parasites and the diseases, which they transmit in this country. To better understand the distribution of ticks, the whole country was divided into five agro-ecological zones using Global-Aridity dataset and 108 livestock farms from 9 different districts, covering the semi-arid and the arid agro-ecological zones in Punjab province, were included in the study. Ticks were collected from two randomly selected animals of each ruminant species present at the farm (194 buffaloes, 179 cattle, 80 goats and 18 sheep) and stored in 70% ethanol. Morphological identification of the ticks was subsequently performed using standard taxonomic keys and multikey software, a computer-based polychotomous key. The identification was confirmed by sequencing of a partial fragment from the second internal transcribed spacer (ITS2) and cytochrome oxidase subunit 1 (cox1) genes from randomly selected specimens of each species which proved that the morphological and molecular data are coherent in the identification of the 4 tick species. The prevalence of ticks between the agro-ecological zones was significantly different ($P = 0.037$). There was no farm found without ticks. In total, 3,807 (12 larvae, 1,231 nymphs, 1,303 females and 1,261 males) ixodid ticks representing four different species were collected: *Hyalomma anatolicum* (n = 3,021, 79.3%), *Rhipicephalus microplus* (n = 715, 18.8%), *Hyalomma dromedarii* (n = 41, 1.0%), and *Rhipicephalus turanicus* (n = 30, 0.9%). *Rh. microplus* was the predominant species in the semi-arid zone, whereas *Hy. anatolicum* was the most abundant tick species in the arid zone. *Hy. dromedarii* and *Rh. turanicus* were found only in the arid zone. In all the districts, multiple tick species were found except Multan district, where only *Hy. anatolicum* was present. The overall proportion of tick-infested ruminants was 78.3% (369/471), and was significantly different ($P < 0.001$, $\chi^2 = 126.9$) among animal species. It was highest in cattle (89.9%), followed by buffaloes (81.4%), goats (60%) and sheep (11.1%). The median tick burdens recorded (43 ticks per animal, ranged from 27-67) were significantly different among the animal species ($P < 0.001$) and were highest in cattle (median =

58), followed by buffaloes (median = 38), goats (median = 19) and sheep (median = 4.5). In large ruminants, older animals carried more ticks than younger animals (buffalo, $P = 0.020$; cattle, $P = 0.002$). It was observed that female animals had higher tick burdens than male animals (buffalo, $P = 0.002$; cattle, $P < 0.001$; goat, $P = 0.014$; sheep, $P = 0.02$). The intensity of infestation was significantly lower in indigenous animals as compared to exotic ($P < 0.001$) and crossbred cows ($P < 0.001$), while the difference was not statistically significant between crossbred and exotic cattle ($P = 0.11$). The majority of the animals were infested with a single tick species (90.5%), while only a few were infested with multiple species (9.5%).

After identification, the ticks were divided into 405 (*Hy. anatolicum* = 300, *Rh. microplus* = 89, *Hy. dromerdarii* = 9, *Rh. turanicus* = 7) tick pools (semi-arid zone = 113, arid zone = 292) and screened by RLB assay for the presence of DNA of 41 tick-borne pathogens, i.e. *Anaplasma*, *Ehrlichia, Babesia, Theileria* and *Rickettsia* species. Out of total 405 tick pools, DNA from at least one tick-borne pathogen was found in 148 (36.5%) pools. Among the positive pools, 94 (63.5%) had a mixed infection with two or more (ranging from 2 to 5) tick-borne pathogen species with 18 different combinations, whereas 54 (36.5%) pools were infected with single tick-borne pathogen species. The overall prevalence estimates of tick-borne pathogens in Punjab were significantly different ($\chi^2 = 90.2$, df = 3, $P < 0.001$), and the prevalence of *Ehrlichia* spp. (22.2%) was highest, followed by *Theileria* (9.9%), *Anaplasma* (7.7%) and *Babesia* spp. (2.5%). 11 species of *Rickettsiales*, namely: *A. centrale*, *A. marginale*, *A. ovis*, *Anaplasma* sp. BL099-6, *Ehrlichia* sp. (Multan), *Ehrlichia* sp. ERm58, *Ehrlichia* sp. Firat, *E. mineirensis*, *Ehrlichia* sp. Omatjenne, *R. massiliae* and *R. raoultii,* were identified in four tick species (*Hy. anatolicum, Hy. dromedarii, Rh. microplus and Rh. turanicus*) from two agro-ecological zones in Punjab through genetic analyses of 16S rRNA. Moreover, four *Babesia* species (*B. bigemina*, *B. bovis*, *B. caballi*, and *B. occultans*) and three *Theileria* species (*T. annulata*, *T. ovis*, and *T. orientalis*) were also detected in *Hy. anatolicum* and *Rh. microplus* ticks. The most common tick-borne pathogen was a hitherto uncharacterized species, i.e. *Ehrlichia* sp. (Multan) (18.0%, CI 14.4-22.1), and 16S rRNA gene sequences showed 99% identity to previously described *Ehrlichia* species. Other common tick-borne pathogens were *Ehrlichia* sp. ERm58 (16.3%, CI 22.8-20.3), *Ehrlichia* sp. Firat (16.0%, CI 12.6-20), *Theileria annulata* (6.7%, CI 4.4-9.6), *Anaplasma marginale* (5.7%, CI 3.6-8.4), *T. orientalis* (*buffeli*) (3.5%, CI 1.9-5.7), *E. mineirensis* (3.2%, CI 1.7-5.4) and *A. centrale* (2.7%, CI 1.4-4.8). This study represents the first evidence of the occurrence of *Anaplasma* sp. BL099-6,

Ehrlichia sp. (Multan), *Ehrlichia* sp. ERm58, *Ehrlichia* sp. Firat, *E. mineirensis, Ehrlichia* sp. Omatjenne, *R. massiliae, R. raoultii, B. occultans* and *T. orientalis* in Pakistan. Moreover, we report the first detection of *A. marginale*, *E. mineirensis* and *Ehrlichia* sp. Omatjenne in *Hy. anatolicum* and *R. raoultii* in *Rh. microplus* ticks. Our data showed that *Hyalomma* and *Rhipicephalus* ticks in Punjab were naturally infected with *Rickettsiales*, *Babesia* and *Theileria* species.

Analysis of questionnaire data using univariable and multivariable logistic regression analysis revealed that the absence of rural poultry on farm ($P = 0.005$, OR = 4.4), not using any acaricides ($P < 0.001$, OR = 7.5), traditional rural housing system ($P = 0.007$, OR = 13.1) and grazing ($P = 0.003$, OR = 12.6) were potential risk factors associated with a higher tick prevalence on livestock farms. Attachment site preferences significantly varied by animal species for *Hy. anatolicum* ($\chi^2 = 140.4$, $P < 0.001$) and *Rh. microplus* ($\chi^2 = 77.6$, $P < 0.001$).

It can be concluded that a much broader spectrum of ticks and tick-borne pathogens is present in Pakistan than previously thought, including several potential zoonotic pathogens. In addition, a novel *Ehrlichia* species with 99% sequence identity to the taxon described previously, was identified. It is expected that the outcomes of this study will be useful in the planning of integrated control strategies for ticks and tick-borne diseases in Pakistan.

7 Zusammenfassung

Epidemiologie von Zecken und durch Zecken übertragene Pathogene in den ariden und semiariden agrarökologischen Zonen Pakistans

Zecken und durch Zecken übertragene Krankheiten haben einen großen Einfluss auf die Tiergesundheit und den Lebensunterhalt der Halter von Nutztieren, besonders in Entwicklungsländern. Das Klima Pakistans ist ideal für Zecken, dennoch mangelt es an systematischen Studien, die sich mit diesen Parasiten und den von ihnen in diesem Land übertragenen Krankheiten beschäftigen. Um die Verbreitung der Zecken besser zu verstehen, wurde das Land mit Hilfe von Global-Aridity Daten in fünf agrarökologische Zonen unterteilt. Daten von 108 landwirtschaftlichen Betrieben aus 9 Bezirken sind in diese Studie eingeflossen, womit die ariden und semiariden agrarökologischen Zonen der Provinz Punjab abgedeckt werden. Zecken wurden in jedem Betrieb von zwei zufällig ausgewählten Tieren jeder vorkommenden Wiederkäuerart gesammelt (194 Wasserbüffel, 179 Rinder, 80 Ziegen und 18 Schafe) und in 70% Ethanol gelagert. Die morphologische Bestimmung der Zecken erfolgte mit taxonomischen Standardschlüsseln und Multikey Software, einem computerbasierten polychotomen Schlüssel. Diese Bestimmung wurde bestätigt durch Sequenzierung eines Teilfragments des zweiten Internal Transcribed Spacers (ITS2) und der Untereinheit 1 des Cytochromoxidase (cox1) Gens von zufällig gewählten Individuen jeder Art, was die Übereinstimmung morphologischer und molekularer Daten für die Bestimmung der vier gefundenen Zeckenarten bestätigt. Die Verbreitung der Zecken in den verschiedenen agrarökologischen Zonen war signifikant verschieden (P = 0,037). Es gab keinen Betrieb ohne Zecken. Insgesamt wurden 3.807 Schildzecken (12 Larven, 1231 Nymphen, 1303 Weibchen und 1261 Männchen) aus vier Arten gesammelt: *Hyalomma anatolicum* (n = 3021; 79,3 %), *Rhipicephalus microplus* (n = 715; 18,8%), *Hyalomma dromedarii* (n = 41; 1,0%) und *Rhipicephalus turanicus* (n = 30; 0,9 %). *R. microplus* war die häufigste Art in der semiariden Zone, während *Hy. anatolicum* die häufigste Zecke der ariden Zone war. *Hy. dromedarii* und *Rh. turanicus* wurden nur in der ariden Zone gefunden. In allen Bezirken wurden mehrere Arten gefunden, außer in Multan, wo es nur *Hy. anatolicum* gab. Der Gesamtanteil der mit Zecken befallenen Tiere betrug 78,3 % (369/471) und war signifikant verschieden (P < 0,001; χ^2 = 126,9) unter den Tierarten. Am größten war der Anteil bei Rindern

(89,9%), gefolgt von Wasserbüffeln (81,4%), Ziegen (60%) und Schafen (11,1%). Der mittlere Zeckenbefall, 43 Zecken pro Tier, von 27 bis 67 reichend, war signifikant verschieden ($P < 0{,}001$) unter den Tierarten und zwar am höchsten bei Rindern (58), gefolgt von Wasserbüffeln (38), Ziegen (19) und Schafen (4,5). Bei den großen Wiederkäuern hatten alte Tiere mehr Zecken als jüngere (Wasserbüffel $P = 0{,}02$; Rind $P = 0{,}002$). Es wurde ferner beobachtet, dass Weibchen mehr Zecken hatten als Männchen (Wasserbüffel $P = 0{,}002$; Rind $P < 0{,}001$; Ziege $P = 0{,}014$; Schaf $P = 0{,}02$). Die Befallsintensität war signifikant niedriger bei einheimischen Tieren als bei exotischen ($P = 0{,}001$) und Kreuzungsrassen ($P = 0{,}001$), aber der Unterschied zwischen exotischen und Kreuzungen war nicht signifikant ($P = 0{,}11$). Die Mehrzahl der Tiere war nur mit einer Zeckenart befallen (90,5%) und nur wenige mit mehreren Zeckenarten (9,5%).

Nach der Bestimmung wurden die Zecken in 405 (*Hy. anatolicum* = 300; *Rh. microplus* = 89; *Hy. dromedarii* = 9; *Rh. turanicus* = 7) Pools unterteilt (semiaride Zone = 113; aride Zone = 292) und mittels RLB auf die Präsenz von 41 Pathogenen untersucht, d.h. *Anaplasma, Ehrlichia, Babesia, Theileria* und *Rickettsia* - Arten. In 148 von 405 Pools (36,5%) wurde DNA von mindestens einem Pathogen gefunden. Von diesen positiven Pools hatten 94 eine Mischinfektion von zwei bis fünf Pathogenen in 18 Kombinationen, während 54 (36,5%) nur mit einem Pathogen infiziert waren. Die geschätzte Prävalenz der von Zecken übertragenen Pathogene in Punjab war signifikant verschieden ($\chi^2 = 90.2$, df = 3, $P < 0.001$) und die Prävalenz von *Ehrlichia* spp. (22,5%) war am höchsten, gefolgt von *Theileria* spp. (9,9%), *Anaplasma* spp. (7,7%) und *Babesia* spp. (2,5%). Obwohl es nicht statistisch signifikant sein mag, lag der höchste Anteil infizierter Zecken bei *Hy. anatolicum* vor (37,3%), gefolgt von *R. microplus* (34,8%), *Rh. turanicus* (28,6%) und *Hy. dromedarii* (22,2%). Neun Arten der Rickettsiales, im einzelnen *A. centrale, A. marginale, A. ovis, Anaplasma* sp. BL099-6, *Ehrlichia* sp. (Multan), *Ehrlichia* sp. ERm58, *Ehrlichia* sp. Firat, *E. mineirensis*, *Ehrlichia* sp. Omatjenne, *R. massiliae*, *R. raoultii* wurden in den vier Zeckenarten aus zwei agrarökologischen Zonen in Punjab mittels 16S rRNA Analyse identifiziert. Weiterhin wurden vier *Babesia* Arten (*B. bigemina*, *B. bovis*, *B. caballi*, and *B. occultans*) und drei *Theileria* Arten (*T. annulata*, *T. ovis*, and *T. orientalis*) in *Hy. anatolicum* und *Rh. microplus* Zecken gefunden. Das häufigste von Zecken übertragene Pathogen war eine bisher unbeschriebene Art, nämlich *Ehrlicha* sp. (Multan) (18,0%, CI 14,4-22,1) und 16S rRNA Gensequenzen zeigten 99% Übereinstimmung zu bisher beschriebenen *Ehrlichia* Arten. Andere verbreitete Pathogene waren *Ehrlichia* sp. ERm58 (16,3%, CI 22,8-20,3), *Ehrlichia* sp. Firat (16%, CI 12,6-20), *Theileria*

annulata (6,7%, CI 4,4-9,6), *Anaplasma marginale* (5,7%, CI 3,6-8,4), *T. orientalis* (*buffeli*) (3,5%, CI 1,9-5,7), *E. mineirensis* (3,2%, CI 1,7-5,4) and *A. centrale* (2,7%, CI 1,4-4, 8). Diese Studie ist der erste Nachweis von *Anaplasma* sp. BL099-6*, Ehrlichia* sp. (Multan)*, Ehrlichia* sp. ERm58*, Ehrlichia* sp. Firat*, E. mineirensis, Ehrlichia* sp. Omatjenne*, R. massiliae, R. raoultii, B. occultans* and *T. orientalis* in Pakistan. Weiterhin ist es der erste Nachweis von *A. marginale*, *E. mineirensis* and *Ehrlichia* sp. Omatjenne in *Hy. anatolicum* and *R. raoultii* in *Rh. microplus* - Zecken. Unsere Daten zeigen, dass *Hyalomma* und *Rhipicephalus* Zecken in Pakistan auf natürliche Weise mit *Rickettsiales*, *Babesia* and *Theileria* - Arten infiziert sind.

Umfrageanalysen mittels univariabler und multivariabler logistischer Regessionsanalyse zeigten, dass die Abwesenheit von Geflügel auf den landwirtschaftlichen Betrieben ($P = 0{,}005$; OR = 4,4), das Nichtverwenden von Akariziden ($P < 0{,}001$; OR = 7,5), die traditionelle Bauweise ($P = 0{,}007$; OR = 13,1) und Weidehaltung ($P = 0{,}003$; OR = 12,6) potenzielle Risikofaktoren sind, die im Zusammenhang mit einem hohen Zeckenvorkommen auf den Betrieben stehen. Die Stellen, an denen sich die Zecken vorzugsweise anhefteten, unterschieden sich je nach Tierart für *Hy. anatolicum* ($\chi^2 = 140{,}4$; $P < 0{,}001$) and *Rh. microplus* ($\chi^2 = 77{,}6$; $P < 0{,}001$).

Schlussfolgernd kann man sagen, dass das Spektrum an Zeckenarten und Pathogenen in Pakistan größer ist als bisher angenommen und einige mögliche zoonotische Pathogene enthält. Außerdem konnte eine neue *Ehrlichia*-Art mit 99% Sequenzübereinstimmung zur Gattung identifiziert werden. Es ist zu erwarten, dass die Ergebnisse dieser Studie für die Planung ganzheitlicher Strategien zur Bekämpfung von Zecken und durch Zecken übertragener Krankheiten in Pakistan nützlich sein werden.

8 Appendices

8.1 Appendix A: Questionnaire, Sample collection performa, Data entry sheets

Questionnaire for investigation of risk factors associated with high tick infestation

Ticks transmit a greater variety of pathogens than any other group of hematophagous arthropods and may cause zoonoses. Infection by tick-borne pathogens not only results in economic hardship for people relying on livestock for their survival but also can be transmitted to the farmers themselves. Many tick-borne pathogens cause more or less serious diseases in livestock and human population.

During first phase of the study the tick samples will be collected from different areas of Punjab province to estimate the burden of ticks on livestock population of Punjab province and also to study their distribution in different areas.

During the second phase of the study the collected samples will be transported to Germany and identified. After the identification, the samples will be tested for presence of tick-borne pathogens that will highlight the high risk areas for the occurrence of TBDs. These epidemiological and molecular studies will help the administrative authorities in designing effective policies and making quick decisions using minimum resources that will lead to control ticks and tick-borne diseases.

The current study has been designed with the following objectives

1. To study the distribution of various ticks species in different areas of Punjab province.
2. To investigate the risk factors associated with tick infestation in farm animals (only cattle, buffaloes, sheep and goat)
3. To detect the potential tick-borne pathogens present in different areas of Punjab province

Reference No./ID _____ Date:____.____. 2013

Personal Information

District	_____
Union council and village	_____
Name of the farmer	_____
Address	_____
Contact No.	_____
Location	X- Coordinate Y-Coordinate
Farm type:	☐ Rural (Traditional) ☐ Semi-commercial ☐ Commercial

Part 1. Farm information

1. What is the size of your farm in hectar? _____

2. How many people live on your farm?

Category	Number
No. of men over 18 years	
No. of women over 18 years No. of children over 5 years	
No. of children up to 5 years	

3. How many animals are currently living on your farm?

Animal species	**Number**
Cattle	
Buffalo	
Sheep	
Goat	
Dogs	
Cats	
Chickens	
Turkey	
Other poultry: Please specify:	
Horses	
Donkeys	
Other animals: Please specify:	

4. Why do you keep ruminants on your farm (*tick one or more*)?

☐ For own supply

☐ As additional source of income

☐ Main source of income

☐ Other, please specify, ...

5. What is your level of education?

☐ Intermediate school

☐ Elementary school

☐ No schooling

☐ Other, please specify, ...

Part 2. Ticks related information

6. During which months do you observe the ticks at your farm?

Month	Ticks occur	Highest infestation
January		
February		
March		
April		
May		
June		
July		
August		
September		
October		
November		
December		

7. Do you use any medicine for the control of ticks?

☐ Yes (*Move to question* 8)

☐ No (*Move to question 10*)

☐ Don't know (*Move to question 0*)

8. Which acaricide(s) do you use, how do you apply them (injectable, oral, topical) and in which month(s) did you use them during the last 12 months (e.g. March, May and August)?

Name of acaricide	Species treated	Form of application	Months of application

9. Do you treat the animals in different groups according to their age? (*move to question 0*)

☐ Yes

☐ No

10. Please indicate any reasons, why you do not use acaricides (tick one or more):

☐ They are too expensive

☐ I do not believe in their effect

☐ I use alternative treatment methods

☐ I do not have access to acaricides

☐ Ticks do not occur

☐ It is normal that animals have ticks

☐ Animals do not seem to suffer from the ticks

☐ Other reasons, please specify:
_____

☐ I don't know

11. Do you use any other strategy for tick control?

☐ Yes, please specify:
_____

_____

☐ No

12. Do you feel any change in the behavior of tick positive animal?

☐ Yes, please specify what changes you observe:
_____

_____

☐ No

13. Do you observe the effect of ticks on milk production?

☐ Yes

☐ No

Part 3. Herd management

14. Does any veterinary officer or para-veterinary staff visit your farm?

☐ Yes

☐ No (*move to question 0*)

15. When does the veterinary officer or para-veterinary staff visit your farm (*tick one or more*)?

☐ On a regular basis; please specify approximate time intervals (e.g. every month, once a year):

_____

☐ If animals are sick or unusual mortalities occur

☐ If animals are to be vaccinated

☐ Other reasons, please specify:

_____

_____

_____

16. Please indicate for each ruminant species, what housing facilities you have?

B	C	S	G	Housing type
☐	☐	☐	☐	Closed sheds
☐	☐	☐	☐	Open houses
☐	☐	☐	☐	Only a roof structure as weather protection
☐	☐	☐	☐	Other, please specify: _____
☐	☐	☐	☐	None (*move to question 19*)

17. What material are the housing facilities mentioned above made of? If housing materials differs between species, please write down the species behind the selected categories.

☐ Hard bricks
☐ Soft bricks
☐ Iron pillars
☐ Wood
☐ Straw
☐ Other, please specify: _____

18. What type of flooring do you have in the housing facilities mentioned above? If floor type differs between species, please write down the species behind the selected categories.

☐ Hard floor (made of bricks or concrete)

☐ Soft floor (soil bedding)

☐ Soft floor plus straw

☐ Other, please specify:

_____

19. Where do you keep the ruminants (cows, buffaloes, goats and sheep) during day time in different seasons? If there are differences between species, please enter the abbreviations for the species in the respective cells.

Season	In housing facilities (see above)	Underneath the trees	Outside the sheds but not under the tree
Dry cold			
Summer			
Monsoon			

20. Is the boundary wall present around the farm?

☐ Yes, around the entire farm
☐ Not around the entire farm, but the area where the ruminants are kept is fully enclosed by a fence or wall
☐ None

21. Do you tether the animals?

	Cows	**Buffaloes**	**Sheep**	**Goats**
During day and night	☐	☐	☐	☐
During the day only	☐	☐	☐	☐
During the night only	☐	☐	☐	☐
Not at all	☐	☐	☐	☐

22. What feeding method do you use (*tick one or more per species*)?

	Cows	**Buffaloes**	**Sheep**	**Goats**
Trough feeding indoor	☐	☐	☐	☐
Trough feeding outdoor	☐	☐	☐	☐
Floor feeding indoor	☐	☐	☐	☐
Floor feeding outdoor	☐	☐	☐	☐
Grazing	☐	☐	☐	☐

23. Which fodder are you providing the animals nowadays?

	Cows	**Buffaloes**	**Sheep**	**Goats**
Compound feed	☐	☐	☐	☐
Cut grass	☐	☐	☐	☐
Grazing only	☐	☐	☐	☐
Other, please specify: ……	☐	☐	☐	☐
Other, please specify: ……	☐	☐	☐	☐

24. Do you store the grass at farm?

☐ Yes, in a closed building

☐ Yes, in an open building

☐ Yes, outside

☐ No

25. Are there any other livestock farms keeping ruminants in surrounding areas?

☐ Yes, please estimate the distance to the closest farm: _____

☐ No

26. How frequently do you dispose animal dung?

☐ Daily

☐ Monthly

☐ Other

☐ Never, please specify what you do with animal dung (e.g. burning, nothing)

27. What kind of vegetation is present at your farm?

☐ Trees, please specify number:

☐ Bushes, please estimate percentage of farm land covered

☐ Grass, please estimate percentage of farm land covered

28. Do you keep the newly purchased animal in quarantine?

☐ Yes

☐ No (*move to question 31*)

29. If yes, then for how many days? _____

30. How many animals died on your farm during the last three months? _____

31. Enlist the signs and symptoms of the animal which died.

Species	Age (M)	Approximate time of death	Symptoms

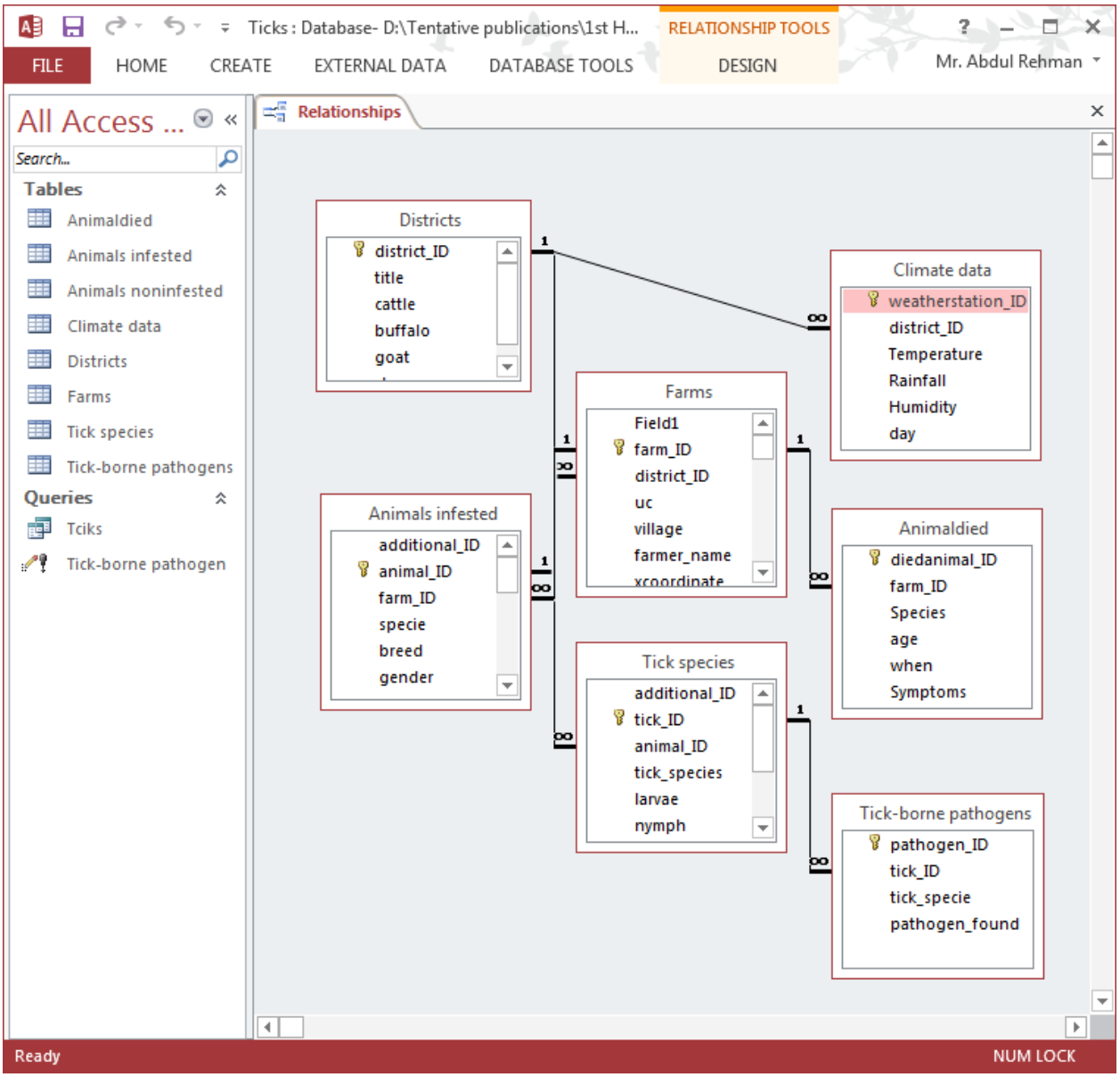
Sample Information for farm ID ____

Sample ID[7]	Species	Breed	Age (M)	Gender	Diseased[8]	Number of ticks collected from different sites					
						Ear	Brisket	Withers	Knees	Udder	Tail
1											
2											
3											
4											
5											
6											
7											
8											

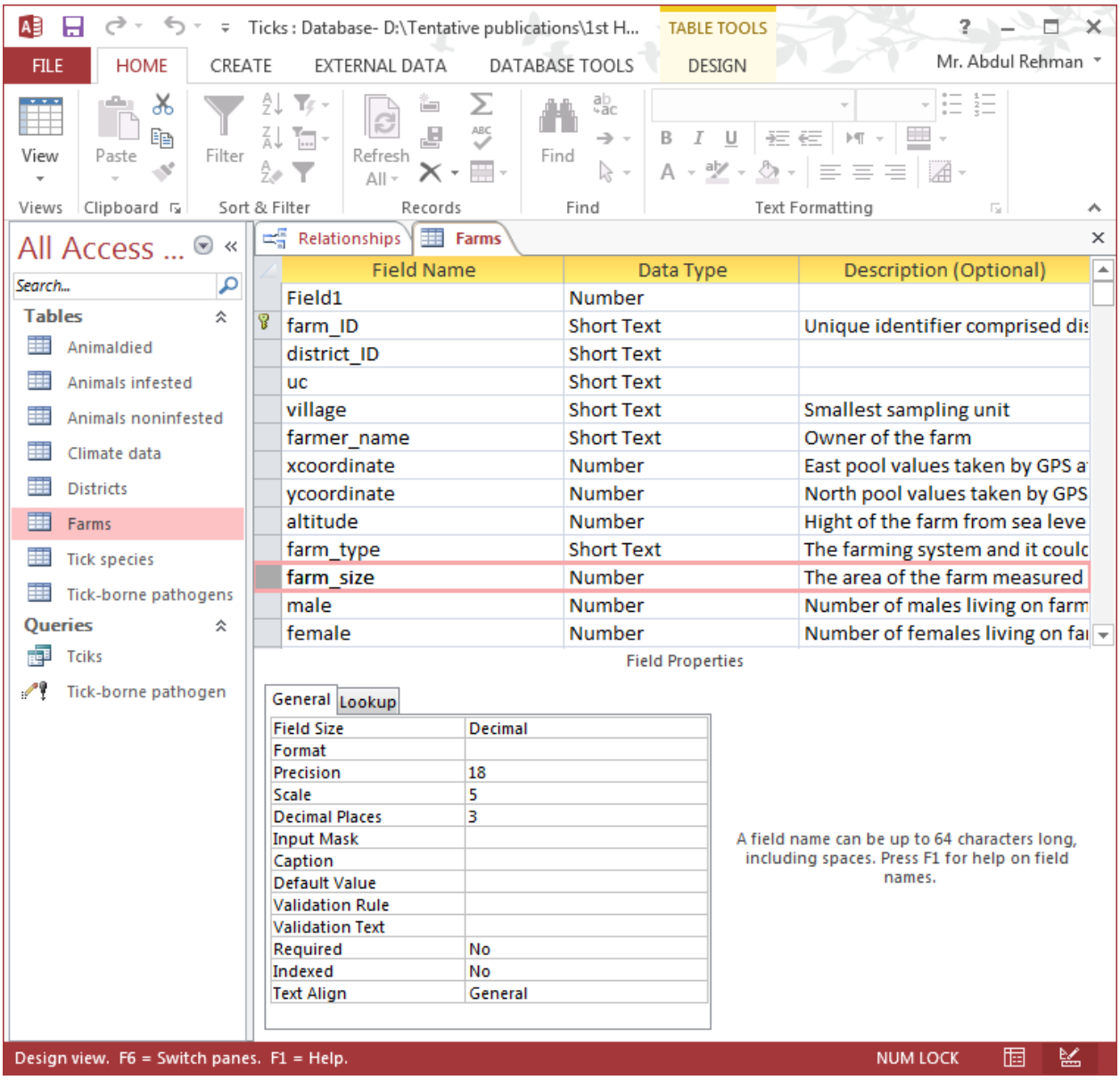
[7] Ticks from an animal will be preserved in 7 different tubes to relate them with the collection site after the identification. Each tube will be labeled with a code which would be the further subdivision of main sample ID, like for sample 1 the tube having ticks from the ear of the animal will be labeled as 1.1 and for dewlap it would be 1.2

[8] Symptoms of diseased animal will be recorded underneath the table with the corresponding sample ID

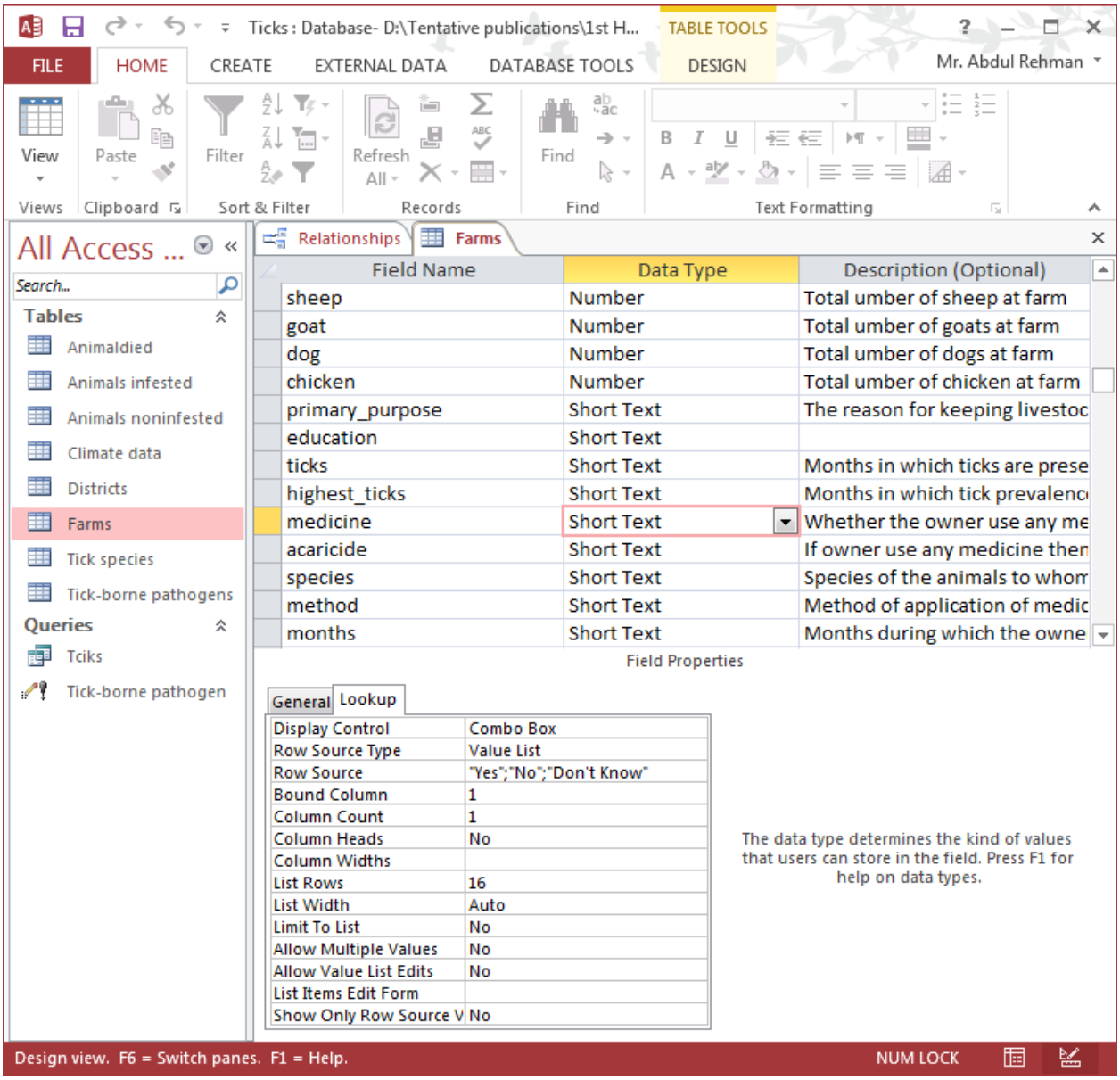
Relationship among different dataset tables in Microsoft Office Access 365 Pro Plus. On the left side, a list of tables is shown, in which the data were stored.



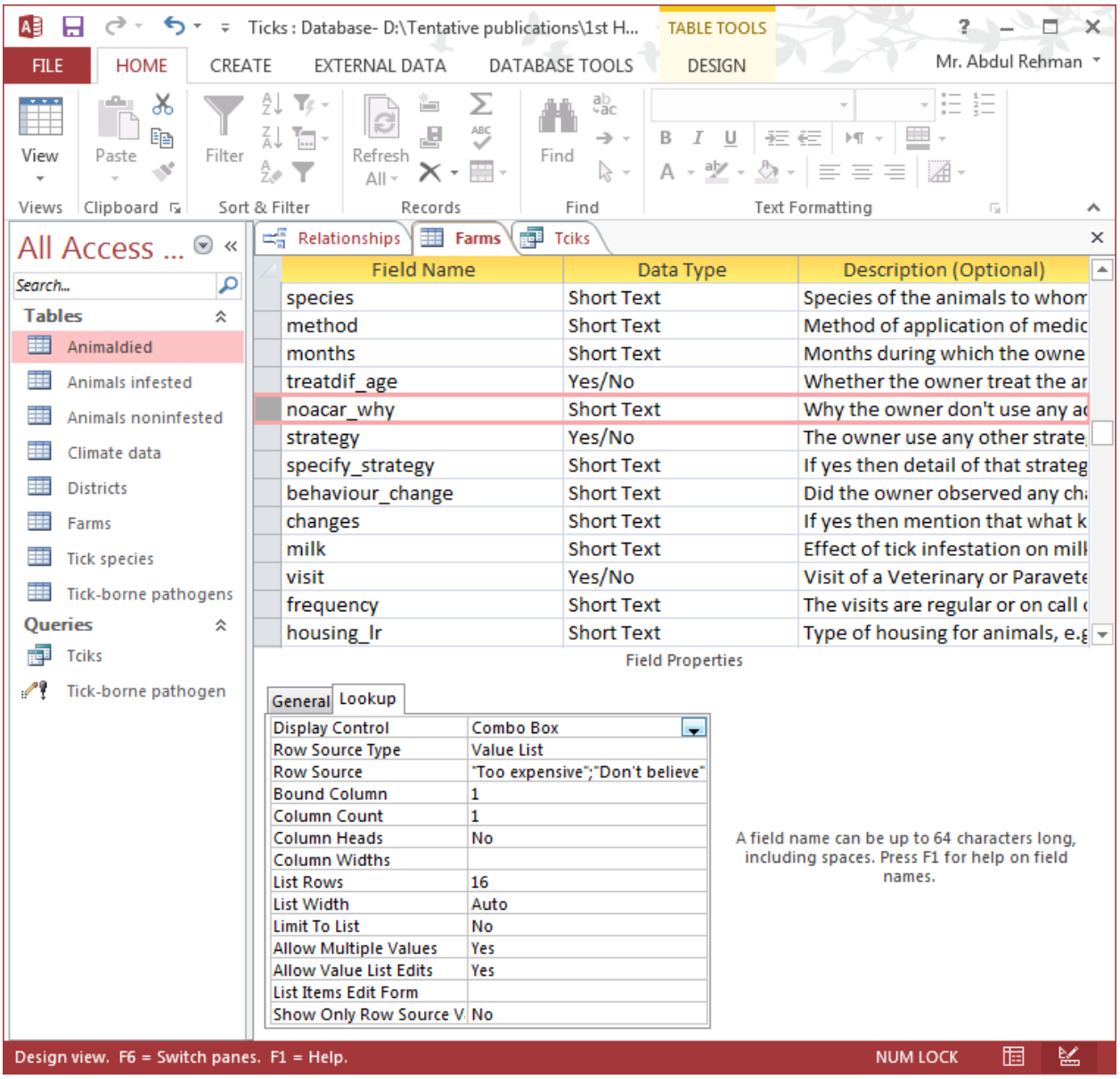
Details of the selected numeric variable (farm size) in design view of the table "Farms" in Microsoft Office Access 365 Pro Plus. The small window towards bottom show how the entry of the variable was specified.



Details of the selected categorical variable (use of medicine against ticks) in design view of the table "Farms" in Microsoft Office Access 365 Pro Plus. The small window towards bottom show how the entry of the variable was controlled for single answer.



Details of the selected categorical variable (use of medicine against ticks) in design view of the table "Farms" in Microsoft Office Access 365 Pro Plus. The small window at the bottom show how the entry of the variable was kept open for multiple answers.



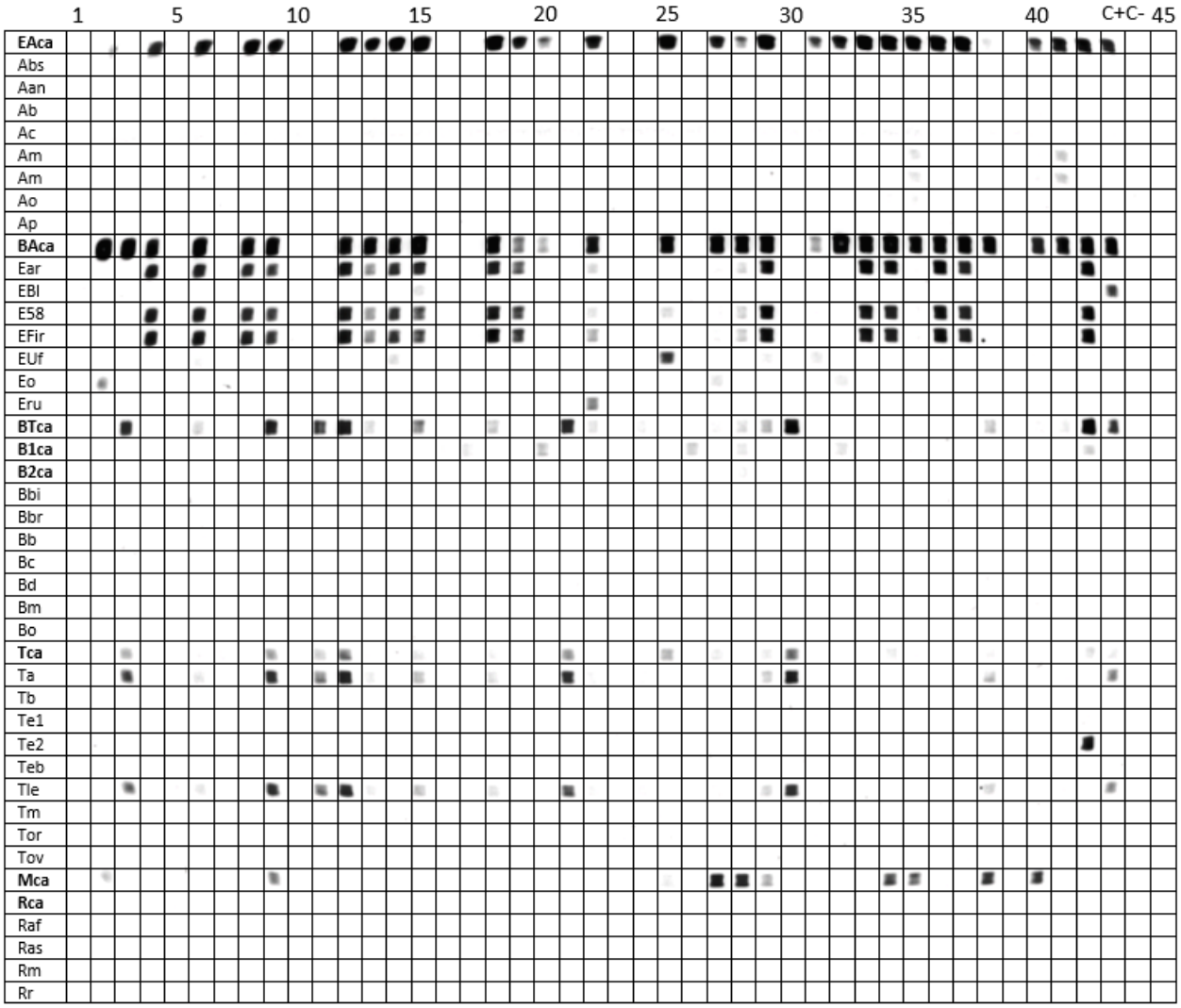
8.2 Appendix B: List of oligonucleotide probes used in RLB and sequence alignment

List of the oligonucleotides probes that were attached on two different membranes and included in the RLB hybridization assay.

Serial No.	Tick-borne pathogen	Oligonucleotides probes sequence (5'→3')
1	*Ehrlichia/Anaplasma catch-all old*	GGGGGAAAGATTTATCGCTA
2	*Anaplasma BSH1 May 2015*	GATTGTTTTCGTAGCTTGCTATGA
3	*Anaplasma Ann M11-2*	CGAACGGATCACTTTTGTAGC
4	*Anaplasma bovis*	GTAGCTTGCTATGAGAACA
5	*Anaplasma centrale new*	TCGAACGGACCATACGC
6	*Anaplasma marginalae old*	GACCGTATACGCAGCTTG
7	*Anaplsma mongolia 3 Eurogentec*	GTATATGCAGCTTGCTGCGTATAC
8	*Anaplasma phagocytophilum new*	G(GA)ATA(GA)TTAGTGGCAGACGGGT
9	*Anaplasma ovis*	ACCGTACGCGCAGCTTG
10	*Anaplasma platys*	GTCGTAGCTTGCTATGATA
11	*Bacteria catch all Eurogentec*	CTACGGGAGGCAGCAGT
12	*Ehrlichia* sp. (Multan)	CTTTGGTATAAATGATTGTTAGTGGC
13	*Anaplasma* sp. BL099-6	GCTTGCTACAAATGTAATTAGTGGC
14	*Ehrlichia* sp. ERm58	CTTTGGTATAAGTAATTGTTAGTGGC
15	*Ehrlichia* sp. Firat	CTTTGGTATAAATAATTGTTAGTGGC
16	*Ehrlichia* sp. UFMG-EV 2	CGGACAATTATTTATAGCTTTTGGC
17	*Ehrlichia* sp. Omatjenne old	CGGATTTTTATCATAGCTTGC
18	*Ehrlichia ruminantium old*	AGTATCTGTTAGTGGCAG
19	*Theileria/Babesia* catch-all old	CTGTCAGAGGTGAAATTCT
20	*Babesia* catch-all 1 old	ATTAGAGTGTTTCAAGCAGAC
21	*Babesia* catch-all 2 old	ACTAGAGTGTTTCAAACAGGC
22	*Babesia bigemina* new	GGGTCTTTTCGCTGGCTT
23	*Babesia* Br May 2015	GACTTGTCCATCTTTTTGGTTCAC
24	*Babesia bovis* new	CAGGTTTCGCCTGTATAATTGAG
25	*Babesia caballi* new	AGAGTGTTTATCGCAGACTTTTGT

26	*Babesia divergens* new	TGACTAATGTCGAGATTGCACTTC
27	*Babesia microti*	G(GA)CTTGGCATC(AT)TCTGGA
28	*Theileria* catch-all new	ATTAGAGTGCT(CT)(AC)AAGCAGGC
29	*Theileria annulata* old	ATTGCTTGTGTCCCTCTG
30	*Theileria buffeli* old	GGCTTATTTCGG(AT)TTGATTTT
31	*Theileria equi A1* new	TTGGCGTTTGTCATCGTTGC
32	*Theileria equi A2* new	GTTGTGGCTTAGTTGGGGCAT
33	*Theileria equi B* new	CTGTATCGTTATCTTCTGCTTGACA
34	*Theileria lestoquardi*	ATTGCTTGTGTCCCTCCG
35	*Therileria mutans* new	GCGGCTTATTTCGGACT(CT)G
36	*Anaplasma marginalae*	GCAAGTCGAACGGACCGTATAC
37	*Theileria ovis*	TTGCTTTTGCTCCTTTACGAG
38	*Theileria orientalis* Br May 2015	GATTTTTTTAT(CT)TTTCCGGATG
39	*Theileria parva* old	GGACGGAGTTCGCTTTG
40	*Theileria separata*	GGTCGTGGTTTTCCTCGT
41	*Theileria taurotragi new*	GGCTTTTTTCGGACGGTTC
42	*Theileria velifera new*	TTCTCCTTTACGAGTTTGGGTCT
43	*Midichloria* catch all	GCGAAATAACAGTTGGAAGCAAT
44	*Rickettsia* catch all	TTTAGAAATAAAAGCTAATACCG
45	*Rickettsia africae*	ACTAATTTTTGGGGCTTGCTC
46	*Rickettsia aeschlimanni*	GGAACCTACCCATCAGTACG
47	*Rickettsia massiliae*	CCGCCACGATATCTAGAAAAATTA
48	*Rickettsia raoultii*	CTAATACCGCATATTCTCTACG

Appendix B

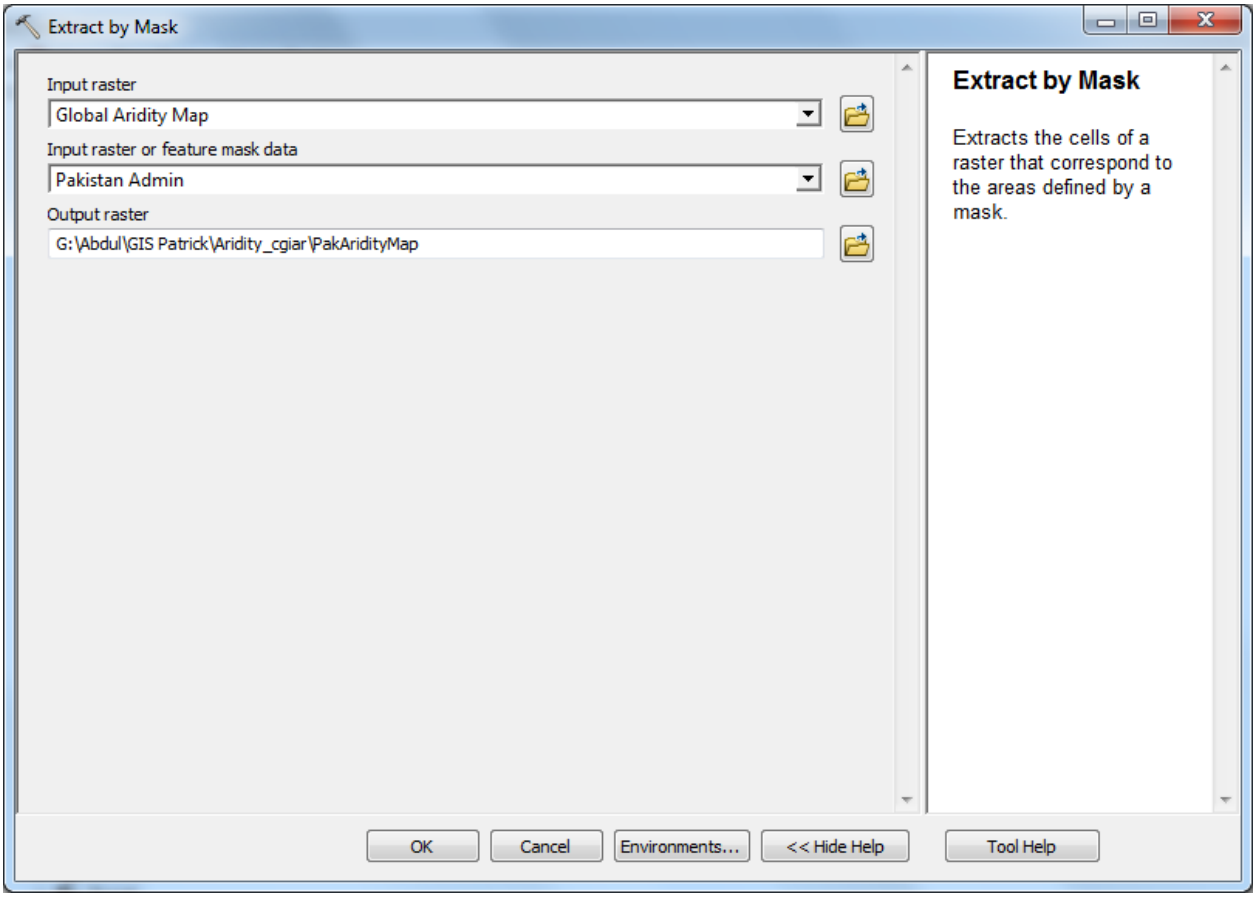


RLB picture after the alignment of the membrane. Each column (from 2nd to 42nd) represents a sample, which was tested for 41 pathogens listed in the 1st column. So, each row represents a pathogen species. The full name of these pathogens along with the sequences used on the membrane are provided in the previous table. Each black spot shows that the sample is positive for the respective pathogen species. Column 43 was loaded with positive control, while the 44th column was loaded with a negative control to verify the reaction. The first and last column in each run were loaded with buffer.

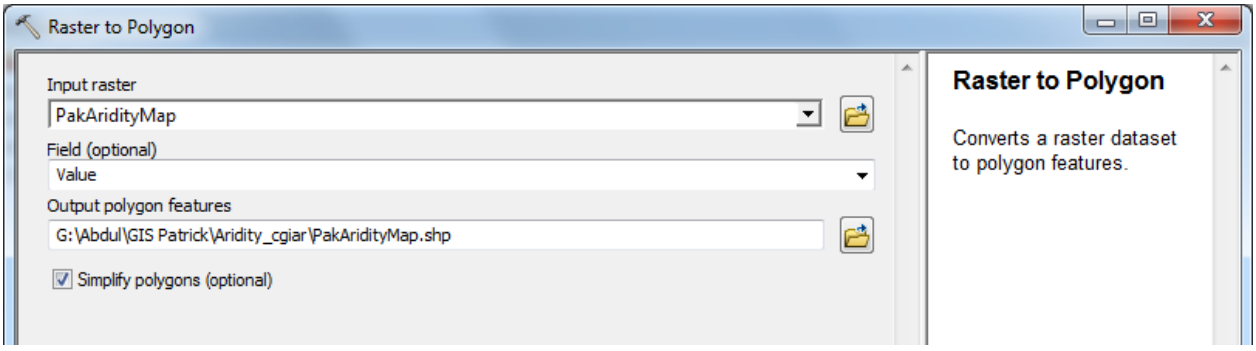
Alignment of *Ehrlichia* sp. (Multan) sequences after ncbi blast in BioEdit software to detect differences relative to the reference sequences of *Ehrlichia* species from other regions.

8.3 Appendix C: Important operations performed in ArcGIS v10.3

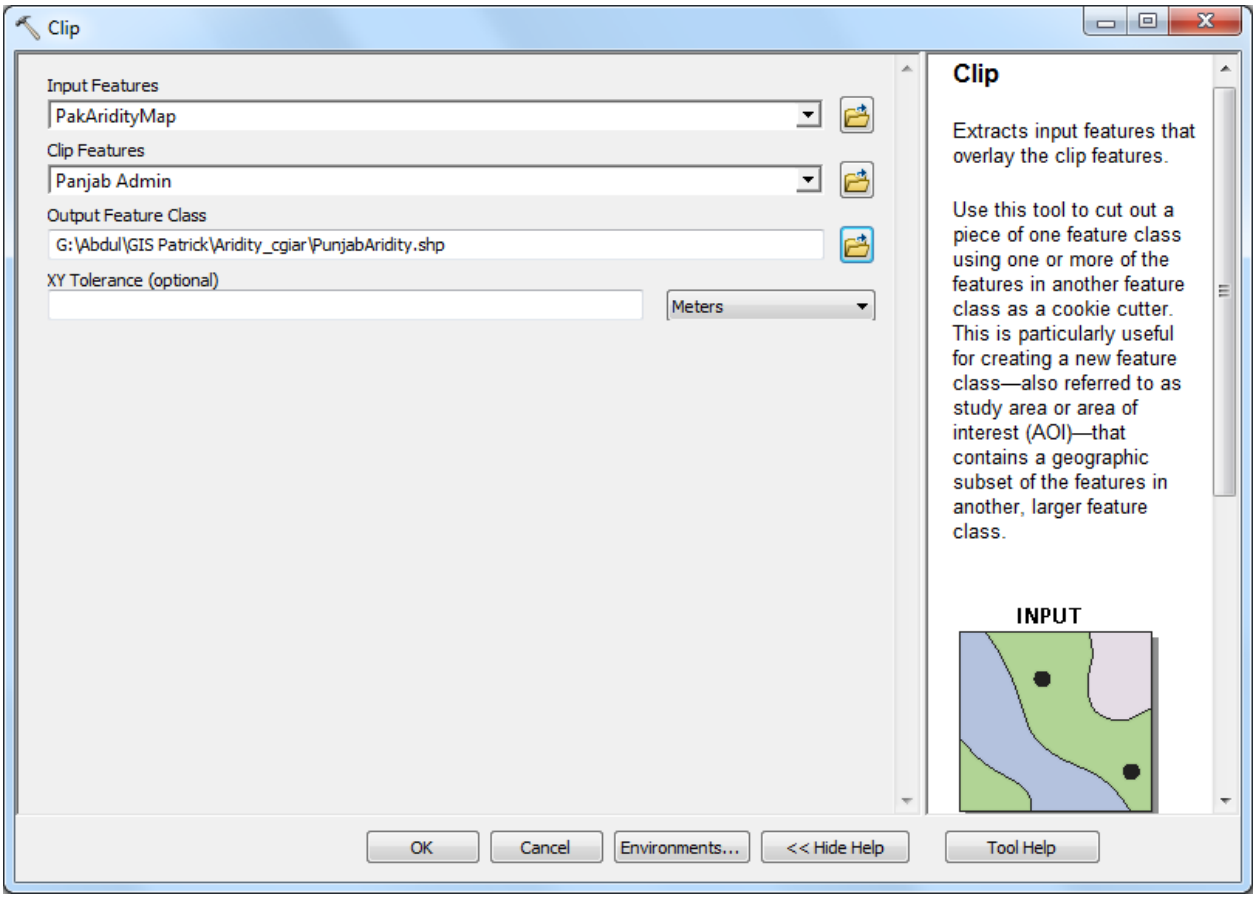
Extraction of cells of the aridity index raster corresponding to administrative boundaries of Pakistan using the Mask function in the "Spatial analyst tool"



Conversion of the raster dataset (PakAridityMap) to a polygon with features commonly known as "Esri shape file" using the "Conversion tool"



Extraction of aridity index polygon corresponding to administrative boundaries of Punjab from "PakAridityMap" using the "Clip" function in Geoprocessing



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A. Rehman, A.M. Nijhof, F.J. Conraths, B. Schauer, C. Staubach. Ticks infesting ruminants on livestock farms in Punjab province, Pakistan. Conference of the professional group on Parasitology and Parasitic Diseases, Stralsund, Germany, 29 June to 01 July 2015 (Oral Presentation).

Acknowledgments

I feel obliged to extend my most humble and naïve gratitude to Almighty ALLAH, who is most compassionate, *gracious*, supreme and caring and whose generous benediction capacitated me to perceive and struggle for the better ideas of life. I send salutes and blessings with all respect to the Holy Prophet "Muhammad" (May peace and blessings of ALLAH be upon him and his descendants), the intercessor, the eloquent and the illustrious who endlessly remains a source of guidance and knowledge for humanity as a whole the whole mankind.

I take benefit of this auspicious opportunity to express with great pleasure, my deepest and intense sense of indebtedness to my dear and respected supervisor Prof. Dr. Franz J. Conraths. His expert advising, untiring support with anything, anytime, anywhere, excellent patience, persistent guidance during the whole span of this program, valuable and prompt feedback with intellectual criticism made it all possible for me to successfully accomplish this work.

I feel a momentous serenity in transcribing my sincere thankfulness to Dr. Ard Menzo Nijhof, Dr. Carola Sauter-Louis, Dr. Birgit Schauer, Dr. Christoph Staubach, Mr. Patrick Wysocki and other scientists at FLI and Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität, Berlin for their close watch, continuous patience, excellent guidance and valuable suggestions without the help of which this effort would never become so comprehensive and apt.

I would like to express my sincere gratitude to the veterinary staff and farmers' community of the respective districts for their help in sampling and data collection. Their names are left from mentioning here only due to dearth of space otherwise their contribution is no less than others in value and magnitude.

I have a great sense of obligation to reverend Dr. Vehale Amin Jaffar for his continuous support in analysing the samples and to Dr. Waseem Shaukat for acquiring weather data from Pakistan Meteorological Department. I am also thankful to Dr. Christoph Krull and Dr. Sarah Knorr for the translation.

Special thanks are due to the Higher Education Commission (HEC) Government of Pakistan and German Academic Exchange Service (DAAD) for providing me with a PhD fellowship.

Acknowledgments

Acknowledgements are due to Pakistan meteorological department (PMPD) for providing weather data, ESRI Inc. for providing ArcGIS, R for statistical software package and Mendley for providing the free reference manager.

Above all, I must acknowledge my indebtedness to my beloved family, particularly to my dearest parents, who not only encouraged me to take this challenging opportunity, supported me continuously, especially during the hard time of my accident, prayed for my endless success, but also trusted on me and presented special endurance and kindness all the way throughout my life. They made me into who I am today. Thanks to you all for being in my life. Here, I would like to pass on my special and everlasting prayers for all of you. I owe you this work. I owe this work to them.

Shortcomings, which may be many in any undertaking of this proportion, remain mine.

Abdul REHMAN

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Dissertation mit dem Titel „Epidemiology of Ticks and Tick-borne Pathogens in the Semi-arid and the Arid Agro-ecological Zones in Pakistan“ selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe. Keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) wurde in Anspruch genommen. Niemand hat von mir mittelbar oder unmittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation am Institut für Epidemiologie des Friedrich-Loeffler-Institutes fertiggestellt. Die vorliegende Arbeit wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Berlin, den 14.12.2016

Abdul REHMAN