Ticks and Tick-borne Diseases of Bovines in Pakistan

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DEDICATION

I dedicate this thesis to my beloved parents, wife and children (Muhammad Ayaan and Muhammad Affan).

SUMMARY

Ticks and tick-borne diseases (TTBDs) impact the health and production of livestock in tropical and subtropical countries, such as Pakistan, where the majority of the rural population is dependent upon production animals for their livelihood. Several tick species are known to infest bovines in Pakistan, and can transmit various tick-borne diseases (TBDs), including anaplasmosis, babesiosis and theileriosis. Despite the substantial adverse socioeconomic impact of TTBDs, little is known about the epidemiology of these diseases in Pakistan. This thesis – comprising six chapters – aimed at investigating some key epidemiological aspects of TTBDs in bovines from different agro-ecological zones (AEZs) of Pakistan.

In Chapter 2, a participatory epidemiological (PE) study was conducted to investigate the major bovine health and production (BH&P) constraints in five AEZs of Pakistan. The findings revealed that TTBDs were among the major BH&P constraints, according to both farmers as well as animal-health professionals (AHPs). Besides providing an insight into the farmers' knowledge about BH&P constraints, this study identified important knowledge gaps among farmers about risk factors and modes of transmission of major infectious disease constraints, including TBDs. This study led to subsequent epidemiological investigations of TTBDs of bovines in Pakistan using molecular methods.

In Chapter 3, morphological and molecular tools were applied in a cross-sectional investigation of bovine ticks from five AEZs of Pakistan. Results revealed a high tick prevalence (46.1%) both in cattle (*Bos indicus* and *Bos taurus*) and water buffaloes (*Bubalus bubalis*); the five tick species identified belonged to two genera, *Hyalomma* and *Rhipicephalus*, with *Hyalomma* (*Hy.*) *anatolicum* – a known vector of a zoonotic disease, Crimean-Congo haemorrhagic fever – have the highest prevalence. This study provided the first genetic characterisation of *Rhipicephalus annulatus* and *Hy. hussaini* from cattle in the Indian subcontinent. The findings of this study highlighted the need to conduct large-scale epidemiological investigations of TBPs in both ticks and bovine populations.

In Chapter 4, the use of a novel, microfluidic real-time PCR revealed a high prevalence of 12 potentially pathogenic and two endosymbiotic microorganisms in ticks of bovines from five AEZs of Pakistan. This study demonstrated the presence of mixed infections with up to five microorganisms in a single tick. Additionally, it provided the first evidence

of the occurrence of several novel and potentially zoonotic pathogens in ticks in Pakistan. Furthermore, ticks from cattle had a higher prevalence of TBPs than those from buffaloes. Similarly, prevalence rates of TBPs in ticks varied among different age groups of bovines. These findings illustrated the advantages of using the novel microfluidic tool for epidemiological investigations of TBDs.

Overall, this thesis has significantly advanced the knowledge of TTBDs of bovines in Pakistan and emphasises the need for further, intensive research on the epidemiology, ecology and population genomics of TTBPs. It also highlights the advantages of using advanced molecular tools to gain deep insights into interactions among vector, microbiome, mammalian host and the environment in the near future. Such insights should contribute toward designing improved methods for the integrated control of TTBDs in Pakistan and elsewhere.

DECLARATION

I do hereby declare that this thesis:

I. Comprises only my original work towards the PhD, except where explicitly stated otherwise in the text or acknowledgements;
II. Due acknowledgement has been made in the text to all other materials used; and
III. The thesis is less than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices

_____

Abdul Ghafar

04 September 2020

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PUBLICATIONS AND PRESENTATIONS

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CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Food security is one of the global challenges affecting almost two billion people worldwide (FAO et al., 2019). The livestock sector plays a pivotal role in global food security by providing means of employment and livelihood, transport, traction, soil productivity and social status (FAO, 2018). Meat, milk, eggs, wool and leather are major commodities obtained from livestock, with milk from cattle and buffaloes as the single most important agricultural product (FAO, 2018).

Globally, infectious diseases are a major constraint to livestock production, particularly those caused by endo- and ecto-parasites (Perry and Randolph, 1999; Rajput et al., 2006; FAO, 2018). For example, ticks are the main ecto-parasites that limit livestock productivity the most (Uilenberg, 1992) by causing direct losses to dairy and beef cattle and associated industries due to hide damage, and indirect losses through the transmission of more than 100 tick-borne pathogens (TBPs) (de la Fuente et al., 2008; Artigas-Jerónimo et al., 2018). Tick-borne zoonoses (TBZ) are also a serious threat to human populations worldwide and include several important emerging diseases (Nicholson et al., 2009; Clow et al., 2019). Tick-borne diseases (TBDs) have often been neglected due to their endemic nature as compared to epidemic diseases like Zika virus disease and Dengue fever (Charrel et al., 2018). However, now the significance of TBDs has made them fit into the 'one world, one health' adage (Randolph, 2009).

Ticks and tick-borne diseases (TTBDs) are common in tropical and sub-tropical countries such as Pakistan, where ~ 80% of the world's cattle population inhabits (de Castro, 1997). The livestock sector plays a vital role in promoting socio-economic development in rural areas of Pakistan. Approximately eight million families are involved in livestock production, representing 35-40% of the annual income to small-scale farmers (Government of Pakistan, 2020). In the last decade, the contribution of livestock has increased steadily in the agriculture sector of Pakistan, contributing 60.6% to this sector in 2019-2020 (Government of Pakistan, 2020).

Pakistan is the world's third-largest producer of milk, and more than 97% of which is produced by a large bovine population, including 49.6 million cattle (*Bos indicus* and *Bos taurus*) and 41.2 million water buffaloes (*Bubalus bubalis*) (Hemme, 2018; Government of Pakistan, 2020). Dairy animals are reared in four milk production systems: (1) rural subsistence smallholdings; (2) rural

market-oriented smallholdings; (3) rural commercial dairy farms; and (4) peri-urban commercial dairy farms (Thomson et al., 1997). Small-scale dairy farms (rural subsistence and rural market-oriented) with less than 10 animals per herd comprise more than 90% of the bovine population in Pakistan, mainly in provinces of Punjab and Sindh (Zia et al., 2011).

The development of the livestock industry in this country is hampered by recurrent outbreaks of infectious diseases, including TTBDs (Wynn et al., 2006; reviewed by Jabbar et al., 2015). The bovine population is exposed to heavy infestation with several species of ticks predominantly belonging to two genera, *Hyalomma* and *Rhipicephalus* (Rehman et al., 2017) as well as TBDs such as anaplasmosis (caused by *Anaplasma* (*A.*) *marginale* and *A. centrale*), babesiosis (*Babesia* (*B.*) *bovis,* and *B. bigemina*) and theileriosis (*Theileria* (*T.*) *annulata*) (reviewed by Jabbar et al., 2015).

This chapter aims to: (i) critically review the current knowledge of the biology of ticks and tick-borne pathogens (TTBPs) and associated economic losses; (ii) review the currently available diagnostic methods and preventive measures for TTBDs; (iii) appraise existing knowledge on TTBDs in Pakistan; (iv) review the role of participatory epidemiology in disease surveillance and assessing farmers' knowledge of animals diseases; and (v) identify knowledge gaps and formulate research aims to address some of the identified gaps.

1.2. Ticks

Ticks (Arachnida: Acari) are obligate blood-sucking ecto-parasites of vertebrates (Anderson and Magnarelli, 2008; Nava et al., 2009). The life cycle of ixodid ticks consists of four developmental stages, the egg, and three active parasitic stages, i.e., larva, nymph and adult (male and female) (Randolph and Rogers, 2010). Depending on the type and species of ticks, their life cycle can vary. Ticks' abundance and distribution in a region are affected by an interaction with environmental conditions (e.g. humidity and temperature) and host population (Randolph and Rogers, 2010). For example, in habitats with low humidity, ticks modify their behaviour and spend limited time questing for hosts in the environment (Estrada-Pena, 2015).

1.2.1. Economic losses caused by ticks and tick-borne diseases

Ticks and tick-borne diseases are a major constraint on livestock development and productivity due to losses associated with milk yield, weight gain, reproductive performance (such as late age of the first calving, abortion and infertility) and loss of animals (Uilenberg, 1995). Additionally, the costs incurred on control, prevention, veterinary services, personnel training and research are also high (Steelman, 1976; Brown, 1997; Perry and Randolph, 1999). Although anyone involved in livestock production would recognise the economic significance of TTBDs, there are very few studies on the reliable estimates of such losses.

For instance, the annual losses attributed by TTBDs to global cattle industry were estimated at USD 7 billion in 1979 (McCosker, 1979) which have gradually increased to $ 13.9-18.7 b in 1997 (de Castro, 1997) and $ 22-30 b in 2015 (Lew-Tabor and Valle, 2016). However, actual global losses due to TTBDs would be much higher because these estimates were solely based on the tick *Rhipicephalus* (*Rh.*) *microplus* using the Australian figures and multiplying by the world cattle population at risk of TTBDs (Jongejan and Uilenberg, 2004). Estimates from Africa show annual losses of $ 168 million due to East Coast Fever (Mukhebi and Perry, 1992). Owing to the multifold harmful effects of TTBDs as well as differences in estimation models and lack of reliable data from under-developed regions, it is very challenging to speculate on the true economic losses caused by TTBDs. A list of estimates of economic losses caused by TTBDs is provided in Table 1.1.

Table 1.1. Estimated economic losses associated with ticks and tick-borne diseases

Region	Ticks and tick-borne diseases	Losses per annum (US$ and mortality)	References
Africa	East Coast fever	500,000 cattle	(Miller et al., 1977)
		168 m and 1.1 m cattle	(Mukhebi and Perry, 1992)
Australia	TTBDs	4 m (Queensland diary industry)	(Jonsson et al., 2001)
		184 m	(Playford, 2005)
Brazil	Cattle tick	3.24 billion	(Grisi et al., 2014)
California	Anaplasmosis	5.2 m (beef cattle)	(Goodger et al., 1979)
Globally	TTBDs	7 b	(McCosker, 1979)
		13.9-18.7 b	(de Castro, 1997)
		22-30 b	(Lew-Tabor and Valle, 2016)
India	Tick worry	57.2 m	(McLeod and Kristjanson, 1999)
	TTBDs	498.7 m	(Minjauw and McLeod, 2003)
	Babesiosis	57.2 m	(McLeod and Kristjanson, 1999)
	Tropical theileriosis	800 m	(Brown, 1997)
Latin America	Anaplasmosis/ Babesiosis	875 m	(Brown, 1997)
Pakistan	Ticks	211.41 (per farm)	(Ashfaq et al., 2015)
	Tropical theileriosis	74.98 (per animal)	(Rashid et al., 2018)
		0.02 m (Holstein Friesian dairy)	
Tanzania	TTBDs	364 m and 1.3 m cattle	(Kivaria, 2006)
	Anaplasmosis	48.1 m	
	Babesiosis	45.8 m	
	Heartwater	22.4 m	
	East Coast fever	247.70 m	
Turkey	Tropical theileriosis	598,133 (per two years)	(Inci et al., 2007)
USA	Anaplasmosis	300 m	(Brown, 1997)
Zimbabwe	Heartwater	6.4 m	(Meltzer et al., 1996)
		5.6 m	(Mukhebi et al., 1999)

1.2.2. Taxonomy of ticks

Among four families of ticks (Argasidae, Ixodidae, Nuttalliellidae and Deinocrotonidae) (Guglielmone et al., 2010; Guglielmone and Nava, 2014; Peñalver et al., 2017), the family Ixodidae (hard ticks) includes almost all of the tick species of major veterinary and public health significance, including approximately 714 species representing 14 genera (*Amblyomma, Anomalohimalaya, Bothriocroton, Cosmiomma, Cornupalpatum, Compluriscutula, Dermacentor, Haemaphysalis, Hyalomma, Ixodes, Margaropus, Nosomma, Rhipicentor* and *Rhipicephalus*) (Barker and Murrell, 2003; Guglielmone et al., 2010; Guglielmone and Nava, 2014). Some members of the family Argasidae (soft ticks) are also of medical and veterinary importance, mainly species of *Argas*, *Ornithodoros* and *Otobius* (Horak et al., 2002; Guglielmone et al., 2010; Estrada-Pena, 2015).

1.2.3. External anatomy of ticks

Externally, a tick comprises of three parts i.e. capitulum (gnathosoma) bearing the mouthparts, body (idiosoma) and legs (Balashov, 1972; Wall and Shearer, 2008; Sonenshine and Roe, 2014). The capitulum is attached to the body by the basis capituli. The mouthparts comprise of a pair of four-segmented palps. Medial to the palps lies a pair of sclerotised two-segmented chelicerae armed with sharp digits which are used for cutting the host skin during feeding. The hypostome is a barbed, medially positioned holdfast organ located ventral to chelicerae, and constitutes food canal. Larvae have three pairs of legs while nymphs and adults have four pairs (Anderson and Magnarelli, 2008; Wall and Shearer, 2008). The tarsus of the first leg carries the Haller's organ, which serves as a sensory organ (Balashov, 1972; Sonenshine and Roe, 2014). A genital aperture (gonopore) and anus are present ventrally at the level of 2nd and 4th pair of legs, respectively (Anderson and Magnarelli, 2008; Wall and Shearer, 2008). An unfed tick ranges in length from 2 to 20 mm, while engorged females range from 25 to 30 mm (Wall and Shearer, 2008).

As this thesis is focussed on ixodid ticks only, the rest of this chapter will cover relevant information pertaining to hard ticks.

1.2.3.1. Ixodidae

Ixodid ticks are relatively large and known as hard ticks due to the presence of a chitinous shield (scutum) on their body. Scutum covers the entire dorsal surface of males while only the anterior dorsal surface in females and juveniles. The sclerotised cuticle of many hard tick species possess symmetrical arrangements of coloured patches and are known as ornate ticks while those which lack such patterns are known as inornate ticks (Wall and Shearer, 2008; Sonenshine and Roe, 2014). Some of the ixodid ticks possess a series of grooves, called festoons, on the posterior margins of the body. Hard ticks have an apical gnathosoma. The adult females are slow-feeders and usually take large volumes of blood meals extended over several days and engorge up to 100 times of their body weight (Wall and Shearer, 2008; Sonenshine and Roe, 2014; Estrada-Pena, 2015). Eyes are usually located on dorsal sides of scutum. Sexual dimorphism is usually clear in the adult stages. Male ticks are smaller than females and take comparatively smaller blood meals. Nymph and adult stages possess large pair of respiratory openings (stigmata) posterior to the 4th pair of legs (Wall and Shearer, 2008; Sonenshine and Roe, 2014).

1.2.4. Life cycle of ixodid ticks

All ixodid ticks have a life cycle involving four stages: (i) egg; (ii) larva; (iii) nymph and (iv) adult. Each of the active stages feeds once and moults into the next stage. Ixodid ticks can be categorised into 3-host (Figure 1.1), 2-host (Figure 1.2) or 1-host (Figure 1.3) ticks depending upon the number of hosts involved in their life cycles. Many tick species (such as *Hyalomma* ticks) can undergo all three categories of the life cycle depending upon the available conditions (Apanaskevich and Oliver, 2014).

Most of the ixodid ticks such as *Amblyomma*, *Dermacentor*, *Haemaphysalis* and *Ixodes*, follow a 3-host life cycle. Fully-engorged adult female ticks detach and drop-off from the host and start oviposition in a suitable site (leaf litter, burrows or crevices). The oviposition is controlled mainly by the environmental temperature. All ixodid ticks are mono-gonotrophic which lay thousands of eggs and then die. Larvae hatch from the eggs in the form of 1-2 mm long seed-ticks and start questing (searching for the host) for a small-sized host such as a rodent. After attachment to the host, larvae feed for 4-6 days, drop to the ground and moult to nymphs within 2-3 weeks. Nymphs attach and feed on the second host (usually a medium-sized host such as a rabbit), drop to the ground and

digest blood meal to become an adult. Adult ticks start questing for the third host which is usually a large mammal such as cattle. Once attached to the suitable host, mating occurs, and females take large volumes of final blood meals before dropping to the ground and start laying eggs.

In habitats where a smaller number of suitable hosts are available and/or under prolonged unfavourable conditions, ixodid ticks (almost 50 species) have adopted a 2-host life cycle (Figure 1.2). In a 2-host life cycle, the larval stages remain attached to the same host after feeding and moult to nymphs. Nymphs drop-off after feeding and moult to adult ticks on the ground. *Hyalomma* (*Hy.*) *isaaci* and *Hy. marginatum* are examples of 2-host ticks. In 1-host life cycle (Figure 1.3), ticks remain attached to the same host throughout larval, nymph and adult stages. The adults finally drop-off and lay eggs. *Dermacentor* (*D.*) *nitens* is an example of 1-host ticks (Apanaskevich and Oliver, 2014).

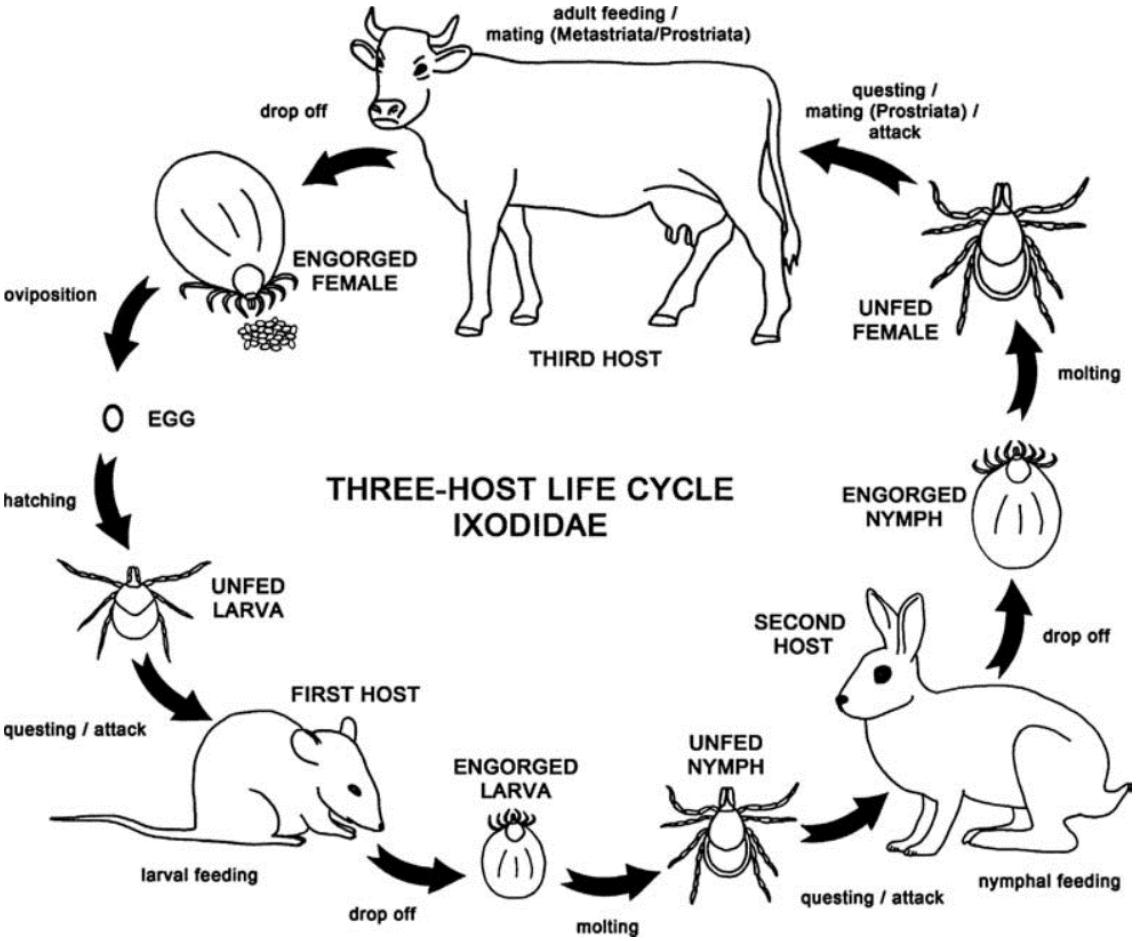

Figure 1.1. Life cycle of a three-host tick (Source: Sonenshine and Roe, 2014)

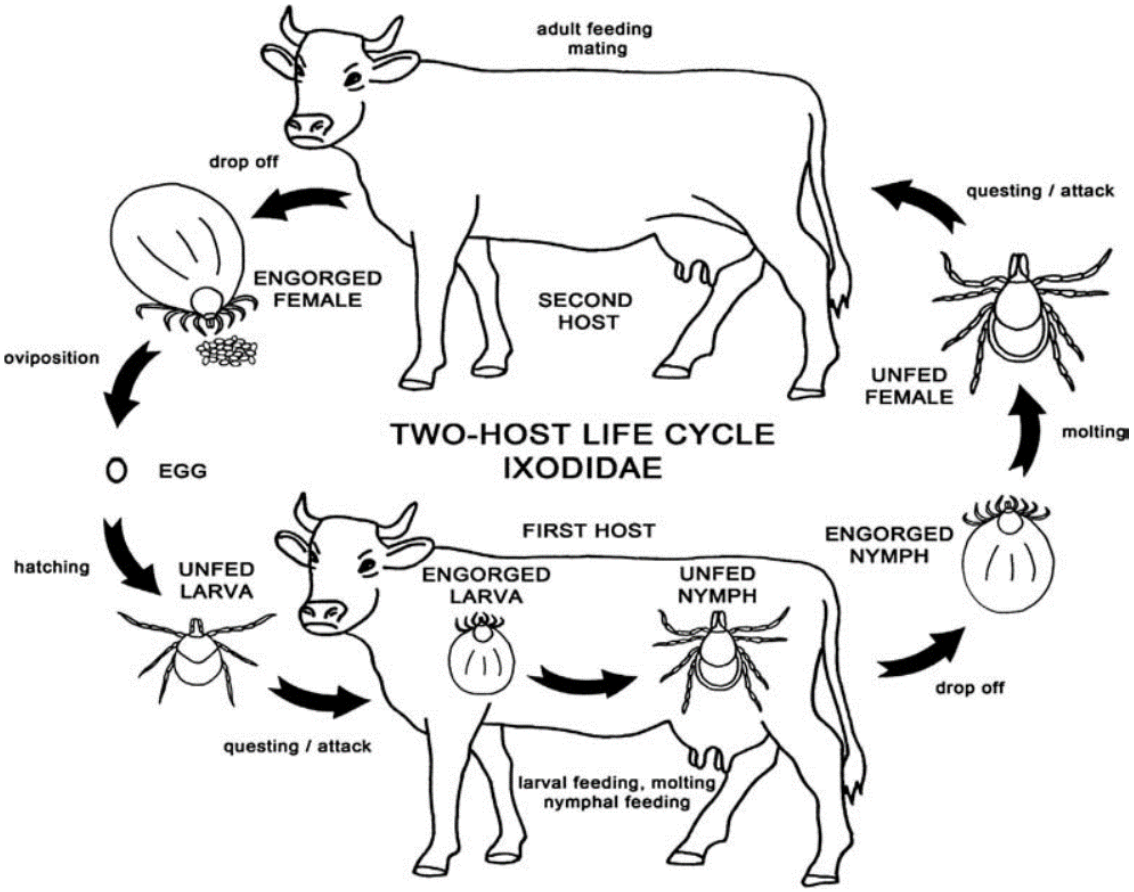

Figure 1.2. Life cycle of a two-host tick (Source: Sonenshine and Roe, 2014)

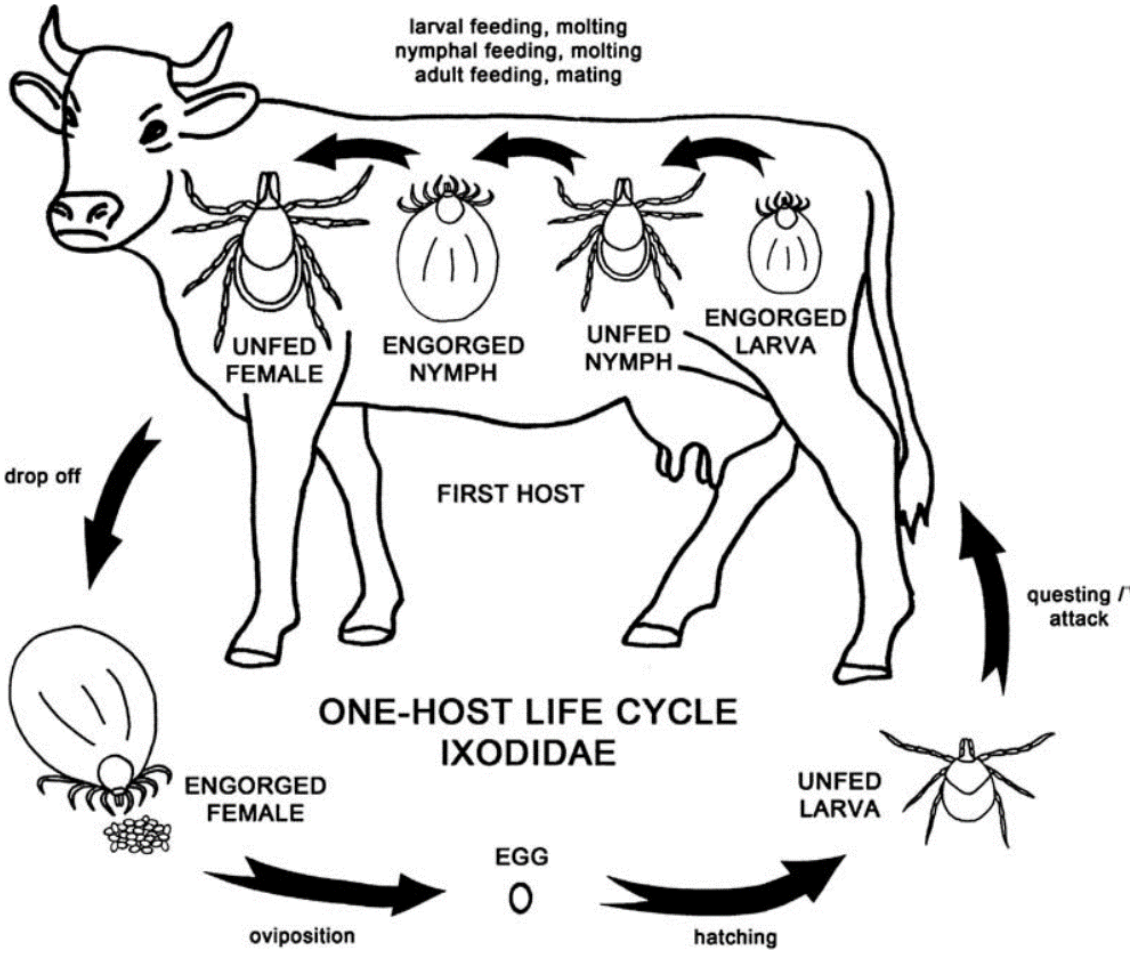

Figure 1.3. Life cycle of a one-host tick (Source: Sonenshine and Roe, 2014)

1.2.5. Effects of ticks on their hosts

Ticks can harm their hosts directly and indirectly. The following sections provide a brief overview of the harmful effects caused by ticks.

1.2.5.1. Direct effects

Direct harmful effects of ticks on their hosts include tick worry, biting stress, toxicosis, skin and hide damage, wounds, loss of productivity (weight gain and milk yield) and anaemia (Rajput et al., 2006; Balashov, 2007). Tick worry is a state of restlessness due to heavy tick infestation, leading to production losses (Drummond, 1976). Heavily-infested animals could also suffer from toxicosis due to several toxins released in the ticks' saliva (Pienaar et al., 2018). Tick-bite paralysis, an acute, progressive and ascending motor paralysis is also common and caused by neurotoxins from 59 ixodid tick species, mainly including *D. andersoni*, *Hy. truncatum* and *Ixodes holocyclus*. Tick bites can cause local pain and an inflammatory response, resulting in the biting stress (Jongejan and Uilenberg, 1994; Atwell, 2016; Janovy Jr and Esch, 2016; Pienaar et al., 2018).

Local inflammatory response and dermal necrosis could damage skin that could result in wounds and secondary bacterial infections. This is particularly important in infestation with ticks bearing long mouthparts such as species of *Amblyomma* and *Hyalomma*. Resulting wounds can become inhabited by the screwworms or other larvae of flies causing myiasis and can also play a role in the spread of bovine dermatophilosis (Wall and Shearer, 2008). Anaemia, due to high volumes of blood loss in heavily infested animals, leads to a reduction in growth rate, weight gain, milk yield and even death. Although the ixodid females enormously increase in size during feeding but the actual blood volume taken is considerably higher as they take concentrated blood meals by releasing back the excess fluid (Jonsson, 2006; Wall and Shearer, 2008). Table 1.2 shows estimated production losses due to TTBDs in bovines.

Table 1.2. Direct and indirect production losses caused by ticks and tick-borne diseases in bovines

Country	TTBDs	Losses	Species/Breed	Trial duration	Reference(s)
Algeria	*T. annulata*	Milk: 2.76 lit per day	Cross-bred	Two months	(Ayadi et al., 2016)
Australia	*Rh. microplus*	Milk: 8.9 ml/tick/day Weight: 1.0 g/tick	Holstein Friesian	15 weeks	(Jonsson et al., 1998)
		Weight: 1.37±0.25 g/tick 1.18±0.21 g/tick	*Bos taurus* *Bos taurus* x *Bos indicus*	Estimates from reported data	(Jonsson, 2006)
	T. orientalis	Milk: 624 lit/head/lactation	Friesian Jersey and cross-bred	305 days	(Perera et al., 2014)
Pakistan	*Hyalomma*	Milk: 1.15 lit/day Butter fat: 1.31%/day	*Bubalus bubalis*	Not given	(Sajid et al., 2007)
Uruguay	Babesiosis	Weight: 45% loss	Hereford	140 days	(Solari et al., 1992)
Zimbabwe	*Rh. appendiculatus*	Milk: 9±4 g/tick/day	Sanga (Mashona)	11 weeks	(Norval et al., 1997b)
	Amblyomma hebraeum	Milk: 6±l0 g Weight: 2.6±1.8 g/tick	Sanga Sanga x zebu (Brahman)		(Norval et al., 1997a)

1.2.5.2. Indirect effects

Ticks are effective vectors of a variety of pathogens of veterinary and medical significance because they infest a wider range of hosts, feed on multiple hosts during lifetime, have resistance to unfavourable conditions and have high reproductive efficiency. Furthermore, ticks remain securely attached to hosts for extended periods of time, which, besides allowing dispersal in a broader habitat, also enhances pathogen ingestion and transmission (Wall and Shearer, 2008; de La Fuente et al., 2017). Transmission of pathogens to co-feeding ticks, transstadial, and transovarian transmission and capacity to carry multiple infections are few other important features which make ticks effective vectors (Wall and Shearer, 2008).

Ticks interact with pathogens and the mammalian hosts in a variety of ways and these complex interactions help to maintain an infected tick population and thus the foci of disease transmission (Estrada-Peña et al., 2015; de La Fuente et al., 2017). Ticks harbour most pathogens in a symbiotic relationship and transmission of these pathogens is highly coordinated with the ticks' feeding cycle (Bonnet et al., 2017; Pollet et al., 2020). Consequently, the list of TBDs is expanding since new TBPs of public-health significance are also emerging such as those causing anaplasmosis, ehrlichiosis, Lyme disease, Crimean-Congo haemorrhagic fever (CCHF) and Bourbon virus disease (de La Fuente et al., 2015; Madison-Antenucci et al., 2020).

The following sections briefly outline the important TBDs transmitted by hard ticks.

1.2.5.2.1. Bacterial diseases

Ehrlichiosis and anaplasmosis are the two economically most important bacterial diseases affecting global livestock production (Uilenberg, 1995; Kirkan et al., 2017). Heartwater or cowdriosis, caused by *Ehrlichia* (*E.*) *ruminatium*, is a major cattle disease in Africa and Caribbean islands (Bram, 1983), transmitted by tick species of genus *Amblyomma* (Uilenberg, 1990). Anaplasmosis, caused by *Anaplasma* (*A.*) *marginale* and *A. centrale*, is widely distributed in tropical and subtropical regions (Uilenberg, 1995; Aubry and Geale, 2011) and is transmitted by at least 17 tick species belonging to genera *Dermacentor*, *Rhipicephalus*, *Ixodes*, *Hyalomma*, and *Argas* (Lew-Tabor, 2016).

1.2.5.2.2. Viral diseases

Ticks are known to transmit at least 50 viruses belonging to 12 genera in animals and humans (Nuttall, 2014; Shi et al., 2018). However, the occurrence of tick-borne viruses (TBVs) of animals is regional compared to haemoprotozoal diseases. Major TBVs of veterinary significance include African swine fever virus, CCHF virus, Dera Ghazi Khan virus, Louping ill virus, Nairobi sheep disease virus, and tick-borne encephalitis virus (Labuda and Nuttall, 2005; Nuttall, 2014).

1.2.5.2.3. Protozoal diseases

Tick-borne protozoa cause the two economically most important livestock diseases, i.e., babesiosis and theileriosis (Uilenberg, 1995). Babesiosis is the most important arthropod-borne disease of cattle worldwide and is mainly caused by *Babesia bovis*, *B. bigemina*, *B. divergens*, and *B. major* (Uilenberg, 2006). It is transmitted mainly by the cattle tick, *Rh. microplus* and also by several species of *Ixodes*, *Hyalomma* and *Haemaphysalis* (de Waal and Combrink, 2006). Human babesiosis is also an important emerging zoonosis worldwide (Gray et al., 2010; Holler et al., 2013). The 2nd major tick-borne protozoa, *Theileria*, causes theileriosis which is an economically important disease of cattle globally and is transmitted by species of *Rhipicephalus* and *Hyalomma* (Schnittger et al., 2012; Vannier and Krause 2012; Perez DE Leon et al., 2014). A detailed account of these and other major bovine TBDs is given later in this chapter.

1.2.5.2.4. Public health significance

Humans are facing an unprecedented increase and spread of infectious diseases, including TBZ, partly due to climate change and the expansion in the range of ticks (de la Fuente et al., 2008; Clow et al., 2019; Dehhaghi et al., 2019). The major TBZ include Lyme disease, Q fever, CCHF, Rocky Mountain Spotted Fever (RMSF), human granulocytic anaplasmosis, human monocytic ehrlichiosis, babesiosis, Louping ill and tick-borne encephalitis (de la Fuente et al., 2008; Baneth, 2014). Lyme disease (caused by *Borrelia burgdorferi*) is a classic example of emerging TBZ infecting more than 400,000 people annually in the US only (Rudenko et al., 2011; Radolf et al., 2012; Davidsson, 2018). This disease was previously present in the temperate regions of the northern hemisphere but now it has spread from North America to Canada (Clow et al.,

2019). The notable increase in the occurrence of TBZ could also be partly due to the availability of sensitive diagnostic tools resulting in higher disease reporting. However, recent reports of CCHF and RMSF epidemics in Turkey and USA, respectively, are the two examples of true increase of TBZ (Maltezou and Papa, 2010; Hubalek and Rudolf, 2012).

1.2.6. Tick collection and estimation of tick burden

Tick collection is a prerequisite to estimate the prevalence and diversity of TTBPs in a zone (Kaiser et al., 1991; Madder et al., 2013), and the resulting data could be helpful to predict risks of TBD outbreaks in a region. Ticks are usually collected directly from several predilection sites on the body of the host, including ear pinna, neck, leg, udder, tail and perineum (Londt et al., 1979). However, ticks can also be collected from the environment (such as pastures, forests, farm-floor) as hard ticks spend more than 90% of their life off the host (Ginsberg and Ewing, 1989). There are four main approaches for the collection of ticks, i.e., (i) flagging or dragging, (ii) trapping using carbon dioxide baits, (iii) collection from the host body (Gray, 1985) and (iv) looking for ticks through the vegetation (Ginsberg and Ewing, 1989). However, each of these methods has merits and demerits. For example, dragging and walking methods may give variable results in the same area due to differences between investigators and CO_2 traps may represent a higher proportion of fast-moving tick species (Ginsberg and Ewing, 1989). Therefore, if possible, a combination of these methods should be used (Terassini et al., 2010; Dantas-Torres et al., 2013; Ramos et al., 2014).

Quantitative data about ticks burden provides useful information about the seasonal variation of ticks, host-parasite interactions, expected losses due to ticks and potentially transmitted pathogens (Kaiser et al., 1991). It is also instrumental in devising control strategies for TTBDs (Mooring and McKenzie, 1995). Tick counting on live animals is time- and labour intensive and requires immobilising the subjects (Yeoman, 1966; MacLeod and Colbo, 1976). An alternative approach is a destructive method which requires killing the animal and counting the separated ticks (Horak, 1982; van Dyk and McKenzie, 1992). Both of these methods are not suitable for use under field conditions. To address challenges faced in the collection of ticks in the field, Mooring and McKenzie (1995) proposed an alternative approach known as a patch-sampling method based on the biology of ticks as they tend to accumulate on certain body parts of the host (Baker and

Ducasse, 1967). This method is quick and does not require specialised equipment or procedure (Mooring and McKenzie, 1995).

1.2.7. Preservation of ticks

The quality of tick specimens determines the successful identification of ticks, extraction of nucleic acid and subsequent detection of pathogens within ticks (Phillips and Simon, 1995). There are different methods used for the storage of ticks such as freezing live ticks at -80°C (Cruickshank, 2002) or refrigerating the killed ticks in 70% or absolute ethanol (Dillon et al., 1996). Boardman (1944) reported that for better delineation, ticks should be killed in a solution of 97 parts of 20% ethanol and three parts of ether and for long term storage, killed ticks should be stored in a solution of nine parts of 75% ethanol and one part of glycerine. Alternative methods include storing ticks in absolute ethanol at -20°C (Guglielmone et al., 2003), 70% ethanol at -80°C (Aktas et al., 2012), 70% isopropyl alcohol (Hoskins, 1991), 70% ethanol at 4°C (Hilpertshauser et al., 2006; Oskam et al., 2017), and at -20°C in sterile tubes (Ebani et al., 2015; Liu et al., 2017). The most frequently used method is storing ticks in 70% ethanol either at room temperature or 4°C (Mtambo et al., 2006).

1.2.8. Control of ticks

The debilitating impact of TTBDs on more than 80% of the world's cattle population and the emerging TBZ have generated greater interest for finding new and effective tick control methods. However, most of the currently used control strategies rely on the traditional use of acaricides and pesticides (Sonenshine and Roe, 2014). History of tick control practice dates back to 1795 when the movement of cattle was restricted by law in North Carolina to stop haemoglobinuria (cause unknown at that time) in local cattle (Walker, 2011). Tick control methods can broadly be categorised into chemical and non-chemical methods.

1.2.8.1. Chemical methods: Acaricides

Use of acaricides constitutes the mainstay of tick control worldwide (Graf et al., 2004). However, there are certain limitations for the use of acaricides such as limited efficacy, acaricide-resistance, high costs and requirement of repeated usage, environmental contamination, withdrawal of residues (particularly in milk and meat) and an expensive and tedious process for the development of new acaricides (Graf et al., 2004). An acaricide can be applied in a variety of ways such as dipping, spraying, pour-on, spot-on, horn-bands, ear-tags, injections and oral administration (Regitano and Prayaga, 2010; Manjunathachar et al., 2014). The history of acaricides use dates to early 1890s when the role of ticks was established as a vector for *B. bigemina* (Angus, 1996). Farmers used to immerse their animals in 'dipping vats' containing a variety of chemicals such as cottonseed oil, fish oil, kerosene, tobacco extract and a mixture of kerosene and sulphur (Mohler, 1906; Angus, 1996).

Arsenic was the first reported chemical compound used as acaricide and was proved very effective in tick control for almost half-century until the resistance emerged (Matthewson and Baker, 1975; George, 2000). Organochlorine compounds were introduced in the 1940s as an alternative, but their usage was limited due to toxicity to host animals and toxic residues (Cobbett, 1947; Maunder, 1949; Manjunathachar et al., 2014). During the 1950s, organophosphates (OPs) and carbamates replaced chlorinated compounds (McDougall and Machin, 1988).

Organophosphates are still being used in some parts of the world (Sonenshine and Roe, 2014) but synthetic pyrethroids (SPs) are currently the most widely used acaricides. Despite reports of resistance against SPs, they are comparatively safer for mammalian hosts and provide effective protection from parasitic arthropods (Graf et al., 2004; Manjunathachar et al., 2014; Sonenshine and Roe, 2014). Amitraz and macrocyclic lactones (avermectins, ivermectin, abamectin and moxidectin) are also being used for tick control but they have longer withholding periods for milk (Cramer et al., 1988; Remington et al., 1997). Spinosad, a tetracyclic-macrolide compound produced by soil-inhabiting bacteria, is the newest weapon against ticks resistant to amitraz and other acaricides (Mertz and Yao, 1990; Graf et al., 2004; Jonsson et al., 2010).

Excessive use of acaricides is one of the key factors for selecting resistant tick populations besides improper dosage, application method and poor quality of acaricides (Sonenshine and Roe, 2014). Tick life cycle also determines the speed of resistance

development as the single-host ticks (e.g. *Rh. microplus*) develop resistance faster than multi-host ticks (Sonenshine and Roe, 2014). The process of resistance development cannot be completely prevented but can be slowed down by reducing the number of acaricide applications, avoiding unnecessary usage, maintaining strict biosecurity and control measures on cattle movement and following proper dosage regimen (Sutherst and Comins, 1979; Roush, 1993).

1.2.8.2. Non-chemical methods

1.2.8.2.1. Grooming

Manual grooming and hand-picking are effective methods for temporary relief from tick infestation and are widely practised in the developing world, particularly by small-scale farmers (Manjunathachar et al., 2014). Hand-picking of ticks is commonly practised during milking, but this method is laborious and needs to be repeated daily to stop pathogen transmission and poses zoonoses threat (Muhammad et al., 2008; Moyo and Masika, 2009; Mondal et al., 2013; Rehman et al., 2017).

1.2.8.2.2. Pasture management

Pasture management through pasture spelling and rotational grazing is an effective strategy in controlling ticks, applied in the developed countries (Walker, 2011). However, this method is not practicable in the developing world where most of the grazing takes place at communal and/or public places (Muhammad et al., 2008; Manjunathachar et al., 2014). Pasture burning through prescribed fires also reduce the risk of TTBDs but tick eggs hidden within the soil may usually escape the effect of burning (Young et al., 1988; Spickett and Fivaz, 1992; Gleim et al., 2019).

1.2.8.2.3. Biological control

Biological control means controlling a pest by introducing a living organism into its environment (Howarth, 1991). Biological control of ticks is becoming an interesting approach (Samish et al., 2004) and several organisms have proven effective, including ants (*Pheidole megacephala*), birds (e.g. oxpecker, poultry), fungi (e.g. *Aspergillus terreus*), insects (e.g. *Ixodiphagus*), plants (e.g. *Stylosanthes*), rodents and shrews.

However, large-scale application of such methods is not possible due to their environmental instability and poor selectivity of target species (Souslby, 1982; Samish and Rehacek, 1999; Manjunathachar et al., 2014).

1.2.8.2.4. Manipulating cattle breeding strategies

Host resistance to ticks represents the only permanent, low-cost method of total tick control as it does not incur the ongoing costs (Burrow et al., 2019). Host resistance against ticks could be innate or acquired and is highly variable between different breeds as well as within the same breed or herd (Marufu et al., 2011; Burrow et al., 2019). Tick resistance can be acquired by mating the tick-resistant breeds (e.g. Sahiwal) with susceptible breeds (e.g. European breeds) (Domingos et al., 2013; Burrow et al., 2019). However, this approach has been neglected due to difficulties associated with the identification of resistance variation at the animal level and the cost associated with selection of cattle resistant to ticks (Burrow et al., 2019).

1.2.8.2.5. Targeting endosymbionts

Ticks are dependent upon endosymbionts for some essential nutrients and therefore removing these microorganisms could deprive ticks of essential nutrients required for their survival. However, this area of tick control remains least exploited so far and offers a promising avenue for future tick control methods (Noda et al., 1997; Ghosh et al., 2007a; Andreotti et al., 2011; Alberdi et al., 2012).

1.2.8.2.6. Immunoprophylaxis

Immunoprophylaxis offers a better alternative to acaricides because vaccines are environment and host-friendly, and less prone to resistance (Jongejan, 1998; Willadsen, 2004; de la Fuente et al., 2007b). Furthermore, tick vaccines are also useful to control pathogen infections and transmission in ticks (Willadsen, 2004; Villar et al., 2017). Despite all these desirable characteristics, no new vaccine has been registered since the first commercial vaccines (TickGARD and Gavac) registered during the early 1990s (de la Fuente et al., 2007a).

The main challenge in the vaccine development is identification and selection of protective antigens. Both commercially available tick vaccines were based on a tick-gut associated 89-kDa glycoprotein known as Bm86 antigen from *Rh. microplus* (Rodríguez et al., 1994;Willadsen et al., 1995). These commercial tick vaccines were registered as Gavac in Cuba in 1993 and as TickGARD in Australia in 1994. Although TickGARD was proven to be effective and resulted in lower dependence upon acaricide in Australia, it is no longer available commercially (Jonsson et al., 2000; de la Fuente et al., 2007a). Gavac has also resulted in a significant reduction of babesiosis and anaplasmosis outbreaks in Cuba, Mexico, Colombia and Brazil (de la Fuente et al., 1999; Valle et al., 2004; de la Fuente et al., 2007a). During the last two decades, the applications of proteomics, reverse genetics and transcriptomics have provided novel means of finding suitable antigens which can provide improved protection against a wider range of tick species and in a broader range of hosts (Villar et al., 2017; de la Fuente, 2018). These approaches are essentially growing existing knowledge about tick-host-pathogen interactions and environmental effects (de la Fuente et al., 2016).

Future tick vaccines would essentially include both tick and pathogen-derived antigens along with the use of traditional tick control methods for integrated and sustainable control of TTBDs (de la Fuente and Contreras, 2015; de la Fuente, 2018).

1.3. Bovine ticks in Pakistan

Tropical to sub-tropical climate and availability of millions of mammalian hosts (primarily ruminants) provide highly conducive conditions for TTBDs in Pakistan (reviewed by Jabbar et al., 2015; Khan, 2019; Government of Pakistan, 2020). Furthermore, more than 90% of the bovine population is owned by small-holder farmers which lack resources for applying proper tick control methods (Zia et al., 2011). To-date, several studies from Pakistan have investigated tick infestation in bovines and reported that more than 80% of the bovine population is exposed to high tick infestation. In Pakistan, the most important bovine tick genera are *Hyalomma* (*Hy. anatolicum*, *Hy. scupense*, *Hy. marginatum* and *Hy. dromedarii*), *Rhipicephalus* (*Rh. microplus*, *Rh. sanguineus*, *Rh. annulatus* and *Rh. turanicus*) and *Haemaphysalis* (*Haemaphysalis* (*Ha.*) *bispinosa* and *Ha. montgomeryi*) (Durrani et al., 2008; Sajid et al., 2008; Karim et al., 2017; Rehman et al., 2017; Ahmad et al., 2019; Ali et al., 2019; Nasreen et al., 2020). However, most of the previous studies from Pakistan used only morphological methods

of tick identification and/or covered small geographical regions of the country without consideration of agro-ecological zones. Therefore, it is possible that a more diverse tick population comprising of several unreported species could be present in the country and need to be investigated using advanced molecular tools. Table 1.3 shows a list of studies conducted on the prevalence of ticks in Pakistani bovines.

1.3.1. Risk factors for bovine tick infestation in Pakistan

Limited knowledge is available about potential risk factors associated with tick infestation in Pakistani bovine population (Sajid et al., 2009; Iqbal et al., 2013; Rehman et al., 2017; Ali et al., 2019). Tick infestation is present throughout the year, but it is usually higher during hot and humid months (June-September) in Pakistan (Rehman et al., 2017; Ali et al., 2019). Lack or less use of acaricidal treatment, due to unavailability and/or poor resources, is a major cause of higher tick infestation in Pakistan (Rehman et al., 2017) and elsewhere.

Notably, the traditional housing system has been considered the most important risk factor for higher tick infestation in Pakistan (Rehman et al., 2017). A traditional farm consists of a closed and an open area where the closed area usually consists of a closed shed with simple roof structure supported by walls made of soft bricks or mud. The outer side of shed walls is also used for dung cakes for fuel. Crevices between wall bricks and dung cakes are one of the most suitable hiding places for ticks. The roof is made of bamboos or similar plant branches and is used for protection from the severe weather (rain, hot and cold) conditions. The sheds are usually completely closed without proper ventilation and sunlight. The floor is usually non-cemented and made of soil or bricks. Such a floor structure also provides good hiding crevices for off-host tick populations (Iqbal et al., 2013; Rehman et al., 2017). Grazing, where practised, is also an important risk factor for higher tick infestation (Ghosh et al., 2007b; Iqbal et al., 2013; Rehman et al., 2017). Furthermore, a recent study from Pakistan reported that farms with backyard poultry were four times less prone to tick infestation as compared to those without poultry (Rehman et al., 2017).

Table 1.3. Studies on the prevalence of different tick species in livestock of Pakistan

Tick Species	Host	Study Area (District, Province)	Duration	Prevalence (%)	Reference(s)
Hy. anatolicum, Rh. sanguineus	Buffalo Cattle	Layyah, Muzaffargarh (P)	NP	51.6 75.1	(Sajid et al., 2008)
Hyalomma, Boophilus, Haemaphysalis, Rhipicephalus	Cattle	Rawalpindi, Multan, Lahore (P)	NP	NP	(Durrani et al., 2008)
Rh. microplus, Rh. annulatus, Hy. aegyptium	Cattle	Dera Ghazi Khan (P)	NP	36	(Ramzan et al., 2008)
Hyalomma, Boophilus, Haemaphysalis, Rhipicephalus	Cattle	Kasur (P)	NP	NP	(Durrani and Kamal, 2008)
Hy. anatolicum	Buffalo Cattle	Layyah, Muzaffargarh (P)	2006-07	47.3 72.9	(Sajid et al., 2009)
Hy. anatolicum, Hy. dromedarii, Rh. microplus, Amblyomma	Buffalo, Cattle	D.I. Khan, Lakki Marwat (KPK)	2009	NP	(Perveen, 2011)
Rh. microplus, Hy. anatolicum	Cattle	Lahore (P)	1996-2000	65.6	(Ahmed et al., 2012)
Hy. marginatum, Rh. microplus	Buffalo	Toba Tek Singh (P)	2010-11	31.2	(Iqbal et al., 2013)
Hyalomma, Boophilus, Amblyomma, Haemaphysalis	Buffalo Cattle	Faisalabad. Jhang, Khanewal (P)	2007	34.0 70.0	(Ali et al., 2013)
NP	Buffalo Cattle	8 Districts (KPK)	2013	22.6 33.4	(Khan et al., 2013a)
Hyalomma, Boophilus,Haemaphysalis, Rhipicephalus	Buffalo	Multan (P)	2009-10	52.5	(Tasawar et al., 2014)
Hyalomma, Boophilus, Haemaphysalis, Rhipicephalus	Cattle	Charsadda, Swabi (KPK)	2010-11	NP	(Ahmad et al., 2014)
Hyalomma, Rhipicephalus, Boophilus, Amblyomma	Buffalo	Khairpur (S)	NP	23	(Soomro et al., 2014)
Hy. anatolicum	Buffalo Cattle	Poonch, Rawalakot (AJK)	2011	51.0 55.5	(Sultana et al., 2015)
Hyalomma, Boophilus	Buffalo, Cattle	Lahore (P)	2012	NP	(Ali et al., 2016)
Hy. anatolicum, Rh. microplus, Hy. dromedarii, Rh. turanicus	Buffalo Cattle	Attock, Gujranwala, Kasur, Okara, Khanewal, Multan, Vehari, Bahawalpur, Rahim Yar Khan (P)	2013-14	81.4 89.9	(Rehman et al., 2017)
Rhipicephalus, Haemaphysalis, Hyalomma	Buffalo, Cattle	Six provinces of Pakistan (P, S, B, KPK, GB, AJK)	2011-12	NP	(Karim et al., 2017)

Rhipicephalus, Haemaphysalis, Hyalomma, Dermacentor, Amblyomma	Buffalo, Cattle	Buner, Mardan, Bannu (KPK)	2015	NP	(Farooqi et al., 2017b)
Hy. anatolicum, D. andersoni, Hy. aegyptium, Rh. microplus	Cattle	Quetta (B)	2013-14	65.6	(Rafiq et al., 2017)
Hyalomma	Buffalo	Rajanpur (P)	NP	44.5	(Khalil et al., 2018)
Rhipicephalus, Haemaphysalis, Hyalomma	Buffalo Cattle	Charsadda, Mardan, Peshawar, Lower Kohistan and Karak (KPK)	2017-18	79.0 87.2	(Ali et al., 2019)
Rhipicephalus, Haemaphysalis, Hyalomma, Dermacentor	Buffalo Cattle	Multan (P)	2017	87.6 63.3	(Ramzan et al., 2020)
Rhipicephalus, Hyalomma	Buffalo, Cattle	Malakand, Buner, Mardan, Swabi, Peshawar (KPK)	2018	NP	(Nasreen et al., 2020)

(AJK, Azad Jammu & Kashmir; B, Baluchistan; GB, Gilgit Baltistan; KPK, Khyber Pakhtunkhwa; NP, Not Provided; P, Punjab; S, Sindh)

1.4. Major tick-borne diseases of bovines in Pakistan

Tick-borne diseases are one of the biggest challenges to bovine production in Pakistan due to a suitable (sub-tropical) climatic conditions for ticks, millions of bovines (mostly malnourished) kept under a traditional farming system with poor husbandry practices, and poor regulation of animal movements (reviewed by Jabbar et al., 2015; Rehman et al., 2019). Moreover, the majority of bovines are owned by small-scale farmers (< 10 animals) who have limited access to extension programs, veterinary services and control measures (if any). All these factors make TBDs a major challenge for livestock production in this country (Afzal, 2010; reviewed by Jabbar et al., 2015; Warriach et al., 2019; Government of Pakistan, 2020).

Bovine population in Pakistan is comprised of indigenous cattle (*Bos indicus*) and water buffaloes (*Bubalus bubalis*), both of which are known to possess some resistance against TTBDs (Robinson, 1982; Pipano and Shkap, 2000; Glass et al., 2005; Bianchin et al., 2007). However, during the last couple of decades, dairy farming has seen an increasing trend towards the import of exotic cattle breeds in this country, particularly at commercial level farms. Furthermore, the small-scale farmers who lack resources for exotic breeds are increasingly crossbreeding their cattle (using government-supplied imported semen) to benefit from the improved genetic potential for higher milk yield at the cost of lower resistance against TTBDS. Major TBDs of bovines in Pakistan include, anaplasmosis, babesiosis and theileriosis (reviewed by Jabbar et al., 2015) and are discussed in the following section. The estimated prevalence of TBDs is given in Table 1.4.

Table 1.4. Estimated prevalence of major tick-borne diseases of bovines in Pakistan

Tick-borne diseases	**Detection Methods**	**Prevalence (%)**		**Range (%)**		**Reference**
		Buffalo	Cattle	Buffalo	Cattle	
Anaplasmosis	Conventional	19.2 ± 4.7	13.2 ± 3.7	3.1-60.0	4.3-60.0	(reviewed by Jabbar et al., 2015)
	Molecular	41.0	NP	NP	NP	
Babesiosis	Conventional	7.5 ± 1.5	5.7 ± 4.4	0.65-29.0	1.0-23.1	
	Molecular	NP	NP	NP	NP	
Theileriosis	Conventional	10.6 ± 3.5	2.65 ± 0.9	0.98-38.3	0.6-5.0	
	Molecular	34.5 ± 15.5	38.7 ± 9.9	19.0-50.0	19.0-66.1	

NP, Not provided

1.4.1. Anaplasmosis

1.4.1.1. Introduction

Anaplasmosis (formerly known as gall sickness) is caused by species of the genus *Anaplasma* (Proteobacteria: Rickettsiales) (Aubry and Geale, 2011; Dantas-Torres and Otranto, 2017a). *Anaplasma* was identified for the first time by Sir Arnold Theiler in 1910 as the causative agent of febrile disease in cattle previously misdiagnosed as babesiosis (Theiler, 1910, 1911; Kocan et al., 2010). Currently, there are seven valid species included in this genus, but the most important species are *A. marginale* and *A. phagocytophilum* - the main causes of bovine anaplasmosis and human granulocytic anaplasmosis, respectively (Aubry and Geale, 2011; Sonenshine and Roe, 2014).

Bovine anaplasmosis is mainly transmitted by ticks (> 20 species) belonging to three genera (i.e. *Hyalomma*, *Rhipicephalus* and *Dermacentor*). However, it can also be spread mechanically through biting flies, surgical instruments such as needles and equipment used for castration, ear tagging, dehorning or tattooing procedures (Kocan et al., 2004; Kocan et al., 2010; Aubry and Geale, 2011; Dantas-Torres and Otranto, 2017a). Transstadial transmission occurs in ticks but there is no evidence of transovarian transmission and the presence of bacteriaemic host is a pre-requisite for maintaining its life cycle (Parrodi and Gualito, 2015; Dantas-Torres and Otranto, 2017a). Male ticks serve as the reservoirs for *A. marginale* and play an important role in its transmission (Kocan et al., 1992a; Kocan et al., 1992b; Ge et al., 1996). In Pakistan, bovine anaplasmosis is caused by *A. marginale* and *A. centrale* and transmitted by *Rh. microplus* and *Hyalomma* spp. (reviewed by Jabbar et al., 2015). Table 1.5 provides a list of studies on bovine anaplasmosis from Pakistan.

Clinical manifestation includes anaemia and jaundice but no haemoglobinuria or haemoglobinaemia is seen (Parrodi and Gualito, 2015). Cattle of all ages are susceptible but those below two years are usually resistant to clinical anaplasmosis (Aubry and Geale, 2011; Lew-Tabor, 2016). After one to nine weeks of the incubation period, the disease may follow a per-acute, acute or chronic course depending upon the host, *Anaplasma* spp. and environmental factors. The per-acute form of infection is very rare and leads to death within hours, whereas in acute form, typical signs include fever, inappetence, rapid drop in milk and weight, icterus, brown discolouration of urine, abortion in pregnant cows and dyspnoea (Lew-Tabor, 2016). During the course of the disease, the number of infected erythrocytes double every 24 hours for up to 10 days and then decrease at a similar rate

(Richey and Palmer, 1990). Irrespective of the age at the time of infection, recovered animals become life-long carriers. *Anaplasma centrale* also causes moderate anaemia in cattle but clinical outbreaks rarely occur (Parrodi and Gualito, 2015; Lew-Tabor, 2016).

1.4.1.2. Life cycle

Ticks transmit the infective stage of *A. marginale* during feeding on a bovine host which subsequently infect the bovine erythrocytes (Figure 1.4). Within the erythrocytes, bacteria multiply and group to make membrane-bound inclusion bodies, each containing four to eight rickettsiae (Kocan et al., 2010). The infected cells are phagocytised by the host reticuloendothelial system and release infective stages for new erythrocytes, leading to anaemia and icterus. The infected erythrocytes are ingested by new ticks and the life cycle continues inside the tick (Kocan et al., 1992a; Kocan et al., 1992b) where *Anaplasma* invades gut cells followed by invading other tissues such as salivary glands (Ge et al., 1996; Marcelino et al., 2012). Within each site in a tick, it is found either as a vegetative form which multiplies by binary fission and/or the dense infective form capable of surviving outside the host. Naïve cattle become infected when infected ticks feed and transfer infective stages through saliva (Marcelino et al., 2012).

Table 1.5. Studies on bovine anaplasmosis from Pakistan

Tick-borne pathogens	**Study area(s) (district, province)**	**Study duration**	**Detection method**	**Prevalence % (n/N)**		**Reference(s)**
				Cattle	Buffalo	
A. marginale	Karachi (S)	1984-85	B/S	60 (30/50)	60 (60/100)	(Haider and Bilqees, 1988)
A. centrale	Hyderabad (S)	1990-91	B/S	7 (7/100)	11 (11/100)	(Buriro et al., 1994)
A. marginale				11 (11/100)	19 (19/100)	
A. marginale	Attock, Islamabad (P)	1999-01	B/S	17.3 (53/307)	12.9 (20/155)	(Khan et al., 2004)
A. centrale	Peshawar (KPK)	2001	B/S	3.86 (11/285)	NA	(Afridi and Ahmad, 2005)
A. marginale				4.2 (12/285)		
A. centrale	Hyderabad (S)	2004	B/S	9.2 (23/250)	8.4 (21/250)	(Rajput et al., 2005)
A. marginale				22 (55/250)	13.6 (34/250)	
A. marginale	Different areas of KPK province	2003	B/S	15.1 (8/53)	26.1 (17/65)	(Talat et al., 2005)
A. marginale	Sargodha (P)	2008-09	B/S	9.7 (34/350)	NA	(Atif et al., 2012b)
A. marginale	Khushab, Rawalpindi, Sargodha (P)	2009-10	B/S cELISA	5.8 (61/1050) 31 (326/1050)	NA	(Atif et al., 2012a, 2013)
A. marginale	Karachi (S)	2011	B/S	NA	9 (9/100)	(Bhutto et al., 2012)
Anaplasma sp.	Bahawalnagar,	2011	PCR-RFLP	NA	41 (116/281)	(Ashraf et al., 2013)
A. marginale	Burewala, Layyah, Multan (P) Kohat, Peshawar (KPK)				17 (20/116)	
Anaplasma sp.	Khanewal (P)	2011-12	B/S	4.1 (34/836)	4.29 (30/700)	(Sajid et al., 2014)
A. marginale	Lahore (P)	2010	B/S PCR	15 (12/80) 32.5 (26/80)	7.5 (6/80) 18.75 (15/80)	(Mushtaqa et al., 2015)
Anaplasma sp.	Charsadda (KPK)	NP	B/S	28 (N=234)	NA	(Khan et al., 2017b)
Anaplasma sp.	Buner, Dir, Mardan, Peshawar, Bannu,	2015	PCR	21.5(103/479)	14.7 (62/421)	(Farooqi et al., 2018)

	Lakki Marwat (KPK)					
Anaplasma sp.	Bahawalpur (P)	NP	PCR	31.8 (47/148)	NA	(Hussain et al., 2017a)
A. bovis	Jhang (P)	2018	Nested	7.78 (35/450)	NA	(Iqbal et al., 2019)
A. phagocytophilum			PCR	2.66 (12/450)		
A. marginale	Chitral, Dir, Malakand (KPK)	NP	PCR	16.3 (30/184)	NA	(Zeb et al., 2020)

(B/S, Blood smear; n, Positive; N, Total; NA, Not applicable; NP, Not provided; RFLP, Restriction Fragment Length Polymorphism; KPK, Khyber Pakhtunkhwa; P, Punjab; S, Sindh)

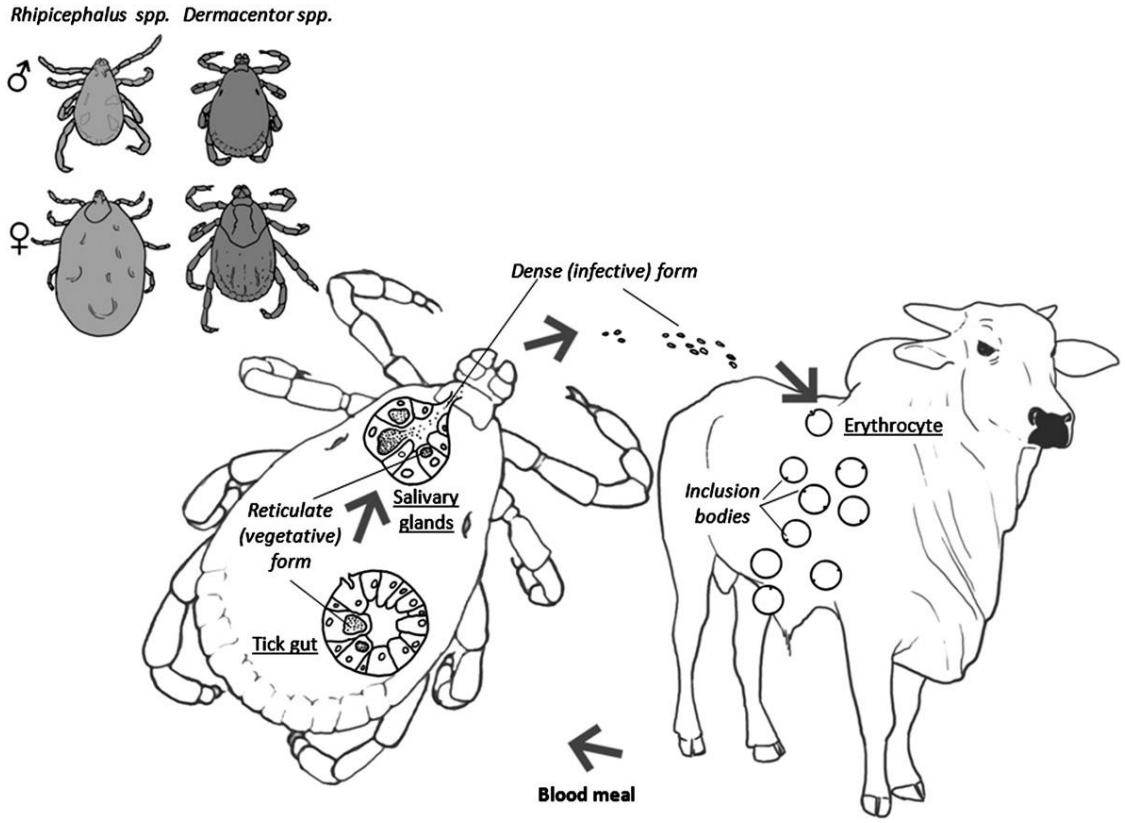

Figure 1.4. Life cycle of *Anaplasma marginale* (Source: Marcelino et al. 2012)

1.4.1.3. Diagnosis

Microscopic examination of thin blood smears is the gold standard diagnostic method for anaplasmosis and is useful during initial 2-6 weeks of infection (Parrodi and Gualito, 2015). Parasites appear as rounded, deeply stained bodies of 0.3-1.0 µm at the margins (*A. marginale*) and centre (*A. centrale*) of erythrocytes (Lew-Tabor, 2016). Contrary to *Babesia*, *Anaplasma* does not accumulate in the peripheral capillaries and thick smears are of less use for its diagnosis. However, light microscopy is of limited value due to lower sensitivity, particularly in carrier animals (Lew-Tabor, 2016). Thin smears from liver, kidney and lungs can also be used for the diagnosis in dead animals (Parrodi and Gualito, 2015).

Serological assays such as indirect enzyme-linked immunosorbent assay (ELISA), competitive ELISA and card agglutination test (CAT), offer efficient and sensitive means of detecting chronically infected animals (Parrodi and Gualito, 2015; Lew-Tabor, 2016). Molecular diagnosis can be made using conventional (c) polymerase chain reaction (PCR), nested (n) PCR and quantitative (q) PCR. The PCR-based methods are highly sensitive for detecting pathogens in carrier animals as nPCR can detect infections with as few as 30 infected erythrocytes per millilitre of blood (Parrodi and Gualito, 2015). Recent developments of high throughput next-generation sequencing and microfluidics-based methods offer sensitive means of exploring population diversity and understanding the host-pathogen interaction in a tick as well as in mammalian hosts (Brinkmann et al., 2019; Sprong et al., 2019).

1.4.1.4. Control methods

Control of bovine anaplasmosis relies on vaccines, chemotherapy, vector control, and maintenance of endemic stability within the herds (Parrodi and Gualito, 2015; Dantas-Torres and Otranto, 2017a). Live vaccines based on *A. centrale* are being used in Australia, Africa, Israel and South America and provide partial immunity against bovine anaplasmosis (Potgieter, 1979; Bock and De Vos, 2001). The vaccinal strain mimics the natural infection cycle and vaccinated animals remain infected for life. However, there have been reports of severe reactions and poor immunity in geographically distinct regions of the world (Bock and De Vos, 2001).

Killed vaccines were developed in the USA and are still being used in some parts of the country (Aubry and Geale, 2011). Attempts have also been made to grow *A. marginale* in tick cell line culture system for use as a vaccine but were of limited success (Munderloh et al., 1996). The novel vaccines should block infection of ticks as well as transmission to the bovine host besides boosting immunity against the pathogen. Various 'omics' tools are being utilised to identify the target vaccine candidates (Aubry and Geale, 2011; Marcelino et al., 2012). Vector control relies on the control of ticks and flies, but it is also laborious and very expensive.

Maintaining herds free of *Anaplasma* could be possible in areas with low levels of infection but needs continuous testing and culling of positive animals, chemosterilisation of carriers and vector control (Reinbold et al., 2010; Aubry and Geale, 2011). Chemotherapy protects cattle for at least eight months and is based on the use of tetracyclines and imidocarb. These drugs are also used for chemosterilisation of carrier animals, but their use is limited due to suspected carcinogenic effect of imidocarb and long withholding periods of tetracyclines (Lew-Tabor, 2016).

The control of TBDs such as anaplasmosis using endemic stability is a well-established concept where all the animals in a herd are infected at an early age and recover without becoming clinically ill (Jones et al., 1968). The early age of calves can be initial few weeks during which the passive immunity is present or early few months during which calves have an age-related resistance of unknown origin (Uilenberg, 1990). For achieving endemic stability against anaplasmosis and other tick-borne diseases, at least 75% of calves in a herd should be infected during the first 6-9 months of age (Jonsson et al., 2012). However, this is a complex process as it requires enough infected tick bites during early age so that calves become immune without undergoing a clinical disease. It is further complicated by the changing population and the number of infected ticks every year.

1.4.2. Babesiosis

1.4.2.1. Introduction

Babesiosis is caused by intra-erythrocytic protozoan species of genus *Babesia* (Apicomplexa: Piroplasmida) (Schnittger et al., 2012; Dantas-Torres et al., 2017). *Babesia* spp. are the second most common mammalian haemoparasites after trypanosomes (Schnittger et al., 2012). Traditionally, *Babesia* species have been classified as small (1.0–2.5 μm) and large (2.5–5.0 μm) *Babesia* when seen under a microscope, however, recent sequencing and phylogenetic advances have demonstrated the presence of more diversity within *Babesia* species (Dantas-Torres et al., 2017; Lempereur et al., 2017).

Bovine babesiosis, Texas fever or more commonly known as red-water fever is economically the most important arthropod-borne disease of cattle worldwide (Smith and Kilborne, 1893; Schnittger et al., 2012; Rathinasamy et al., 2019). It is caused by *B. bovis* and *B. bigemina* in tropical and subtropical countries including Pakistan and is transmitted by *Rh. microplus*; whereas, in temperate regions, it is caused by *B. divergens* and transmitted by *Ixodes ricinus* (Bock et al., 2004; Igarashi and Parrodi, 2014). Economically important *Babesia* species also vary in pathogenicity, distribution and clinical manifestations. For example, *B. bovis* is more pathogenic, whereas *B. bigemina* is most widely distributed (Igarashi and Parrodi, 2014). Clinical infections with *B. bovis* result in fever, respiratory distress, inappetence, anaemia, uncoordinated movements, haemoglobinuria and death. Some animals also show nervous signs caused by the sequestration of infected erythrocytes into cerebral capillaries (Bock et al., 2004). Reduced fertility and abortions in affected bulls and pregnant cattle, respectively, are also common. The recovered animals could remain carriers for years. Clinical manifestations associated with *B. bigemina* infections include an early and massive erythrocytic destruction and haemoglobinuria without any cerebral signs. Parasitaemia level is very high (up to 36%) compared to *B. bovis* (< 1%) and fever is less often seen (Bock et al., 2004; Igarashi and Parrodi, 2014). Clinical signs and parasitaemia in *B. divergens* infections are similar to *B. bigemina* infections (Zintl et al., 2003). Table 1.6 provides a list of studies on bovine babesiosis from Pakistan.

Table 1.6. Studies on bovine babesiosis from Pakistan

Tick-borne pathogens	Study area(s) (district, province)	Study duration	Detection method	Prevalence % (n/N)		References
				Cattle	Buffalo	
Babesia sp.	Karachi (S)	1984-85	B/S	4.2 (4/95)	1.4 (3/219)	(Haider and Bilqees, 1987)
Babesia sp.	Hyderabad (S)	1990-91	B/S	1 (1/100)	1 (1/100)	(Buriro et al., 1994)
Babesia sp.	Attock, Islamabad (P)	1999-01	B/S	0.65 (2/307)	0 (0/155)	(Khan et al., 2004)
B. bigemina	Peshawar (KPK)	2001	B/S	1.75 (5/285)	NA	(Afridi and Ahmad, 2005)
B. bovis				2.80 (8/285)		
Babesia sp.	Kasur (P)	2003-04	B/S	2.5 (5/200)	NA	(Zahid et al., 2005)
B. bigemina	Malakand Agency (KPK)	NP	B/S	5.2 (42/794)	NA	(Ahmad et al., 2006)
Babesia sp.	Malakand Agency (KPK)	NP	B/S	6.6 (73/1100)	NA	(Ahmad and Hashmi, 2007)
B. bigemina	Kasur (P)	NP	B/S	6 (6/100)	NA	(Durrani and Kamal, 2008)
B. bovis				13 (13/100)		
			PCR	3 (3/100)		
				7 (7/100)		
Babesia sp.	Sahiwal (P)	2005	B/S	7.2 (30/415)	NA	(Niazi et al., 2008)
B. bigemina	Sahiwal (P)	2005	B/S	18 (18/100)	NA	(Chaudhry et al., 2010)
B. bovis			PCR	18 (18/100)		
B. bovis	Bahawalnagar, Bhakar, Layyah, Multan, Muzaffar Garh, Vehari (P)	2010	B/S	2.7 (4/144)	NP	(Zulfiqar et al., 2012)
			PCR	17.1 (18/105)	23.1 (9/39)	
B. bovis	Karachi (S)	2011	B/S	NA	3 (3/100)	(Bhutto et al., 2012)
B. bigemina	Sargodha (P)	2008-09	B/S	6.57 (23/350)	NA	(Atif et al., 2012b)
B. bigemina	Khushab, Rawalpindi, Sargodha (P)	2009-10	B/S	4.8 (50/1050)	NA	(Atif et al., 2012a)
Babesia sp.	Karak, Kohat (KPK)	NP	B/S	5.83 (35/600)	NA	(Shams et al., 2013)
			PCR	27.5 (165/600)		
B. bigemina	Charsadda, Swabi (KPK)	2010-11	B/S	19 (19/100)	NA	(Ahmad et al., 2014)
B. bovis				11 (11/100)		
B. bigemina	Peshawar (KPK)	2011	B/S	21.9 (16/73)	0 (0/27)	(Saad et al., 2015)

B. bovis				0 (0/73)	29.6 (8/27)	
			PCR	20.5 (15/73)	22.2 (6/27)	
				12.3 (9/73)	7.4 (2/27)	
B. bovis	Lahore (P)	NP	B/S	6.67 (4/60)	NA	(Hussain et al., 2017b)
			PCR	8.3 (5/60)		
B. bovis	Different zones of KPK	2015	B/S	11.89 (57/479)	8.0 (34/421)	(Farooqi et al., 2017a)
			PCR	10	10	
B. bigemina	Chakwal, Jhang, Faisalabad (P)	2016	Semi-nested PCR	2.4 (11/450)	N/A	(Hassan et al., 2018)

(B/S, Blood smear; n, Positive; N, Total; NA, Not applicable; NP, Not provided; KPK, Khyber Pakhtunkhwa; P, Punjab; S, Sindh)

1.4.2.2. Life cycle

The variability of life cycle among different *Babesia* species is under debate and several important aspects are still unknown. The life cycle is characterised by typical apicomplexan stages, including merogony, gametogony and sporogony (Suarez and Noh, 2011). (Figure 1.5). The infected ticks transfer sporozoites, the infective stages, to the susceptible host while having a blood meal (Dantas-Torres et al., 2017). *Babesia* sporozoites only infect erythrocytes of the vertebrate hosts (Friedhoff, 1988) and develop into trophozoites which divide by binary fission to produce two to eight merozoites. After a few cycles of multiplication, the merozoites are released from ruptured erythrocytes and infect new cells (Mehlhorn and Schein, 1985). Some merozoites assume an ovoid shape and become gammonts or pre-gametocytes (Mackenstedt et al., 1995; Dantas-Torres et al., 2017).

Upon ingestion by the next feeding ticks, gammonts develop into two populations of ray bodies or gametes which subsequently fuse to form diploid zygotes (Golgh et al., 1998). Zygotes infect the tick gut cells and divide by meiosis into elongated and motile kinetes. Kinetes escape gut cells and invade various tissues, including salivary glands and ovaries via haemolymph (Agbede et al., 1986; Mackenstedt et al., 1995). Once inside the salivary glands, kinetes transform into multinucleated sporoblasts which give rise to sporozoites, the infective stage for the next vertebrate host (Mackenstedt et al., 1995). The development of sporozoites starts only after the tick attachment to the host is complete and takes about two to three days in case of *B. bovis* (Riek, 1966) and nine days for *B. bigemina.* Therefore, the transmission of *B. bovis* occurs through all tick stages while the latter is transmitted through nymphal and adult tick stages only (Hoyte, 1961; Potgieter and Els, 1977; Bock et al., 2004).

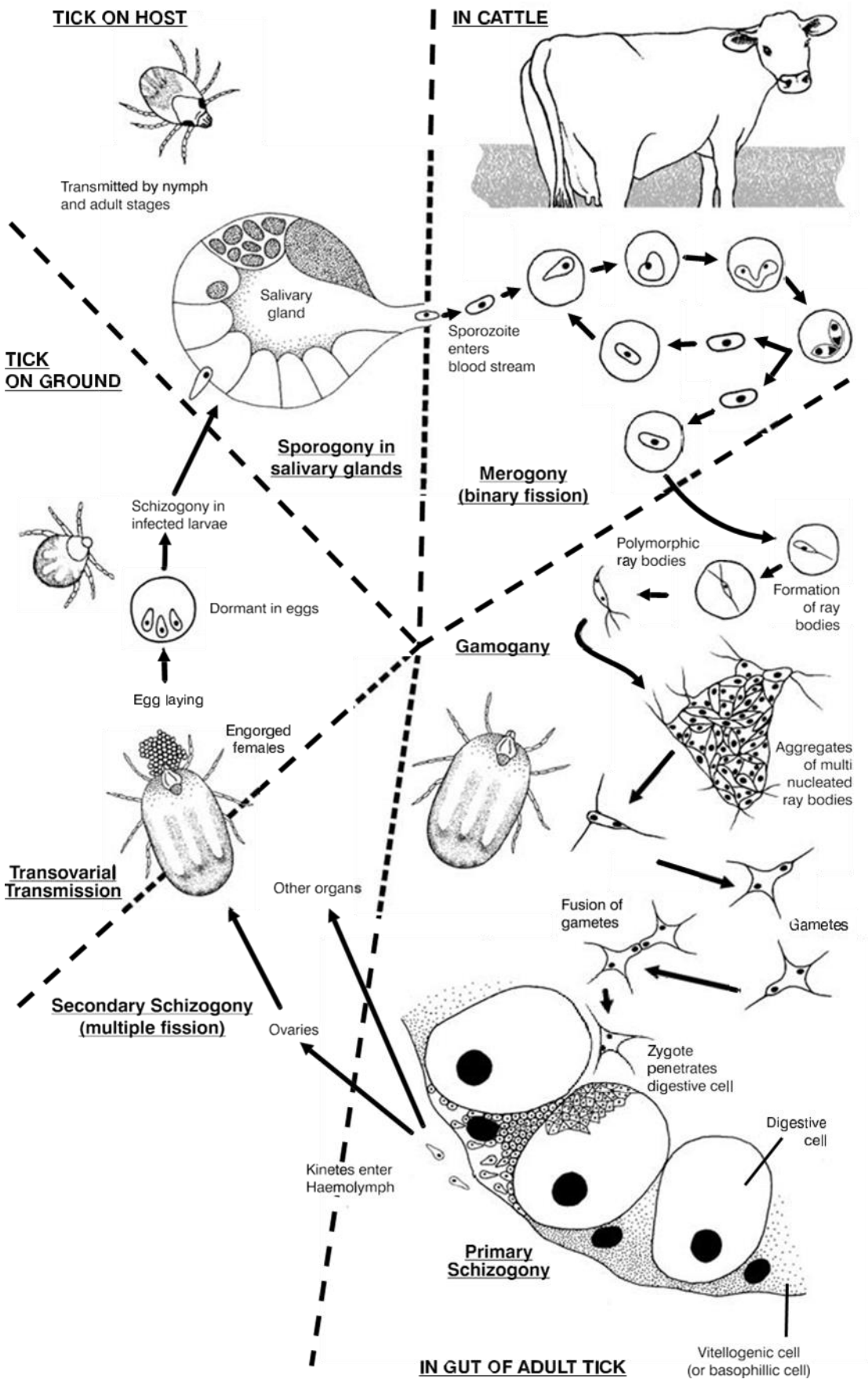

Figure 1.5. Life cycle of *Babesia bovis* (Source: Bock et al. 2004)

1.4.2.3. Diagnosis

Direct microscopic examination of thin and thick smears remains the gold standard and economical method for the detection of *Babesia* infections (Lempereur et al., 2017). Thin films enable differentiation of species whereas thick films increase the test sensitivity (up to 1 parasite in 10^6 erythrocytes) but require expertise and time to interpret and perform, respectively. Furthermore, it is useful in detecting only the acute infections where the parasitaemia level is high but not the carriers or infections with low parasitaemia (Igarashi and Parrodi, 2014). For *B. bovis* diagnosis, fresh blood smears should be made preferably from capillary blood of tail and ear tips while venous blood can be used for *B. bigemina* infections. Post-mortem diagnosis can be made using smears from spleen, brain and kidneys (Igarashi and Parrodi, 2014; Lempereur et al., 2017).

In vitro culture methods have also been applied to demonstrate the carrier infections but not adopted on a large scale (Holman et al., 1993). A number of serological methods, including indirect fluorescent antibody test (IFAT), complement fixation test (CFT), indirect ELISA and competitive ELISA can also be used for the diagnosis of babesiosis. Although ELISA is the most commonly used serological assay, it has not been validated for *B. bigemina* infections because the antibody level declines rapidly and may reduce to negative threshold level within months of infection (Goff et al., 2008; Igarashi and Parrodi, 2014).

Diagnostic assays based on nucleic acid detection are very sensitive and offer efficient means of detecting carrier animals from blood and spleen (Lempereur et al., 2017). These methods include cPCR, qPCR, nPCR, loop-mediated isothermal amplification (LAMP), multiplex LAMP and high-resolution melting (HRM) assay. PCR-based methods can detect the parasitaemia level as low as 1 parasite per 10^9 erythrocytes (i.e. 1000 times higher sensitive than microscopy) (Criado-Fornelio et al., 2009; Wang et al., 2019). The LAMP method is cost-effective and sensitive for the detection of *Babesia* infections under field conditions without requiring sophisticated equipment (Liu et al., 2012). Recent advances in the field of genomics have resulted in the development of highly sensitive methods which could be used in investigating the population dynamics and diversity of *Babesia* spp. (Chaudhry et al., 2019; Glidden et al., 2019; Huggins et al., 2019).

1.4.2.4. Control methods

Control of bovine babesiosis relies on vector control, the use of babesicidal drugs and vaccines and the application of endemic stability concept (Suarez and Noh, 2011). Although the vector control has proven less useful in controlling babesiosis globally, results are remarkable in the USA where it has resulted in the eradication of babesiosis (Pegram et al., 2000).

As discussed in the previous section on anaplasmosis, the concept of endemic stability is also applicable for babesiosis. Young calves are usually resistant to babesiosis for first two months due to passive immunity of colostral origin followed by an age-related innate immunity up to nine months of age (Bock et al., 2004). It has been established that if at least 75% calves are exposed to *B. bovis* infection by 6-9 months of age, they become immune for life-long against the same *Babesia* sp. (Mahoney, 1974; Dalgliesh, 1993). There is also evidence of some level of cross-protection against *B. bovis* due to *B. bigemina* infections but not in the reverse scenario (Bock et al., 2004). This approach, however, cannot be considered reliable because of variations in the environment, host and pathogen properties as well as the requirement of sustained infection rate at the desired level (Bock et al., 2004).

Chemotherapeutic control of babesiosis relies on using two major anti-protozoal drugs i.e. diminazine aceturate and imidocarb (Mosqueda et al., 2012; Gohil et al., 2013). Imidocarb provides protection against acute bovine babesiosis for four to eight weeks depending upon the *Babesia* species (de Waal and Combrink, 2006). However, challenges such as resistant strains and drug residues in meat and dairy products limit the use of these drugs (Zintl et al., 2003; Mosqueda et al., 2012). Recently, new compounds have been identified with anti-protozoal properties and are being tested for their efficacy against apicomplexan parasites (Mosqueda et al., 2012).

Live attenuated vaccines, containing *B. bovis* and *B. bigemina*, have been in use since long in Australia, Africa, South-East Asia, and South America (de Waal and Combrink, 2006; Igarashi and Parrodi, 2014). In Australia, it is prepared as chilled as well as a frozen form containing *B. bovis*, *B. bigemina* and *A. centrale* for the control of cattle tick fever. Another vaccine has also been developed containing an attenuated strain of *B. divergens* (Zintl et al., 2003). However, there are several limitations associated with the use of live vaccines such as short shelf-life, the requirement of cold chain and ability to cause clinical disease in the immuno-compromised animals (Suarez and Noh, 2011). Future control of

babesiosis would rely on the successful development of subunit vaccines containing multiple components from different life stages of *Babesia*, combined with anti-tick vaccines as well as transmission-blocking vaccines (Bock et al., 2004; Gohil et al., 2013; Rathinasamy et al., 2019).

1.4.3. Theileriosis

1.4.3.1. Introduction

Theileriosis is a group of lymphoproliferative diseases caused by intracellular protozoa belonging to the genus *Theileria* (Apicomplexa: Piroplasmida) which affect a wide range of domestic and wild ruminants worldwide (Sivakumar et al., 2014; Nene and Morrison, 2016; Dantas-Torres and Otranto, 2017b). Based on their ability to induce host-cell proliferation, *Theileria* species can be categorised into transforming and non-transforming types (Sivakumar et al., 2014). The two important pathogenic species of *Theileria* which infect bovines are *T. parva* and *T. annulata* (Geysen, 2014; Dantas-Torres and Otranto, 2017b).

Theileria parva causes East Coast fever - an economically important disease of cattle in the eastern and southern Africa, and is transmitted by *Rh. appendiculatus* (Geysen, 2014). *Theileria annulata* causes tropical theileriosis in north Africa, the Mediterranean coastal areas, the Middle East and Asia and is transmitted mainly by *Hyalomma* ticks (Geysen, 2014; Sivakumar et al., 2014). Geographical distributions of both types of bovine theileriosis do not overlap, partly due to the difference in the distribution of their vectors. Other *Theileria* spp. that often cause asymptomatic infections are *T. taurotragi*, *T. mutans* and members of *T. orientalis* complex (Gebrekidan et al., 2020). More recently, clinical outbreaks of theileriosis have been associated with *T. orientalis* complex from the Asia-Pacific region (Gebrekidan et al., 2020). Asian buffaloes are susceptible to tropical theileriosis, whereas African buffaloes possess natural resistance against East Coast fever and serve as reservoirs of infection (Geysen, 2014). In Pakistan, tropical theileriosis is caused by *T. annulata* and is transmitted mainly by *Hy. anatolicum* along with *Hy. dromedarii* and *Hy. marginatum* (reviewed by Jabbar et al., 2015). Table 1.7 provides a list of studies on bovine theileriosis from Pakistan.

Clinical signs of theileriosis include fever, lymphadenopathy, inappetence, loss of body weight, excessive lacrimation and nasal discharge, dyspnoea and death. Anaemia and jaundice may be seen in cases of tropical theileriosis but not in *T. parva* infections.

Tropical theileriosis could also cause abortions in pregnant cows and haemoglobinuria and haemorrhagic diarrhoea prior to causing death in some cases. Recovered animals may remain carriers for years (Sonenshine and Roe, 2014; Dantas-Torres and Otranto, 2017b).

1.4.3.2. Life cycle

The life cycle of the *Theileria* species (Figure 1.6) is very similar to that of *Babesia* species and consists of asexual and sexual phases within the vertebrate and tick hosts, respectively (Bishop et al., 2004; Dantas-Torres and Otranto, 2017b). Sporozoites are injected into the mammalian blood when an infected tick feeds on the susceptible host. Sporozoites infect leuckocytes and develop into the schizont stage within the cytosol and then transforms them to highly proliferative cells. When the host cells are lysed, several merozoites are released which infect erythrocytes and develop into piroplasms. The intra-erythrocytic piroplasmic stage undergoes further multiplication in case of *T. annulata* while limited or no further multiplication occurs in case of *T. parva.* Due to this difference, anaemia is not a feature of *T. parva* infections, whereas it does occur in *T. annulata* infections. Nevertheless, the main clinical manifestation is due to leuckocytic invasion by the pathogens. The piroplasm stage is taken up by the ticks during feeding and the gametes undergo sexual reproduction in the tick gut to produce zygote which further divides to form motile kinetes. The motile kinetes infect tick-gut epithelial cells and migrate to salivary glands via haemolymph. When ticks start feeding, sporogony begins and sporozoites are injected into the next host. Transstadial transmission occurs but transovarian transmission has not been reported in this protozoon (Bishop et al., 2004).

Table 1.7. Studies on bovine theileriosis from Pakistan

Tick-borne pathogens	Study area(s) (district, province)	Study duration	Detection method	Prevalence % (n/N)		References
				Cattle	Buffalo	
Theileria sp.	Faisalabad (P)	1982	B/S	100 (3/3)	NA	(Ashfaque et al., 1983)
Theileria sp.	Hyderabad (S)	1990-91	B/S	3 (3/100)	5 (5/100)	(Buriro et al., 1994)
T. annulata	Faisalabad (P)	1993-98	Signs B/S	79.5 (89/112)	NA	(Muhammad, 1999)
T. annulata	Attock (P), Islamabad	1999-01	B/S	0.98 (3/307)	0.6 (1/155)	(Khan et al., 2004)
T. annulata	Peshawar (KPK)	2001	B/S	1.4 (4/285)	NA	(Afridi and Ahmad, 2005)
T. annulata	Kasur (P)	2003-04	B/S	24 (48/200)-HF 15 (30/200)-J	NA	(Zahid et al., 2005)
Theileria sp.	Lahore (P)	2003	B/S	NA	17 (107/600)	(Durrani et al., 2006)
T. annulata	Kasur (P)	NP	B/S PCR	14 (14/100) 36 (36/100)	NA	(Durrani and Kamal, 2008)
T. annulata	Sahiwal (P)	2009	B/S	38.3 (115/300)	NA	(Qayyum et al., 2010)
T. annulata	Bahawalnagar, Bhakar, Layyah, Multan, Muzaffar Garh, Vehari (P)	NP	B/S PCR	3 (4/144) 19 (28/144)	NP	(Shahnawaz et al., 2011)
T. annulata	Karachi (S)	2011	B/S	NA	2 (2/100)	(Bhutto et al., 2012)
T. annulata	Sargodha (P)	2008-09	B/S	6.7 (24/350)	NA	(Atif et al., 2012b)
T. annulata	Khushab, Rawalpindi, Sargodha (P)	2009-10	B/S	4.8 (50/1050)	NA	(Atif et al., 2012a)
T. annulata	Kohat, Peshawar (KPK)	2010-11	B/S PCR	5.3 (5/95) 33.7 (32/95)	NA	(Khattak et al., 2012)
T. annulata	Okara, Shiekhupura (P)	NP	PCR	66.1 (41/62)	50 (20/40)	(Khan et al., 2013b)
T. annulata	Cholistan (P)	2013-14	B/S PCR	1.9 (5/264) 19.3 (51/264)	NA	(Zaka et al., 2016)
Theileria sp.	Hyderabad (S)	2013-14	B/S PCR	85 (N=300) 85 (N=300)	70 (N=2400) 76 (N=2400)	(Memon et al., 2016)

Theileria sp.	Charsadda (KPK)	NP	B/S	18 (N=234)	NA	(Khan et al., 2017b)
T. orientalis	Jhang, Kasur, Shiekhupura, Vehari (P)	2015	PCR	Nt: 0 (0/50) Im: 15(22/147)	0 (0/49)	(Gebrekidan et al., 2017)
			MT-PCR	Nt: 6 (3/50) Im:24.5(36/147)	6.1 (3/49)	
Theileria sp.	Dera Ismail Khan (KPK)	2016	B/S	14.3 (55/384)	NA	(Khan et al., 2017a)
T. annulata *T. orientalis*	Chakwal, Jhang, Faisalabad (P)	2016	Semi-nested PCR	22.9 (103/450) 3.1 (12/450)	NA	(Hassan et al., 2018)
T. annulata	Chitral, Dir, Malakand (KPK)	NP	PCR	30 (55/184)	NA	(Zeb et al., 2020)

(B/S, Blood smear; n, Positive; N, Total; NA, Not applicable; NP, Not provided; MT, Multiplex; HF, Holstein Friesian; J, Jersey; Nt, Native; Im, Imported; KPK, Khyber Pakhtunkhwa; P, Punjab; S, Sindh)

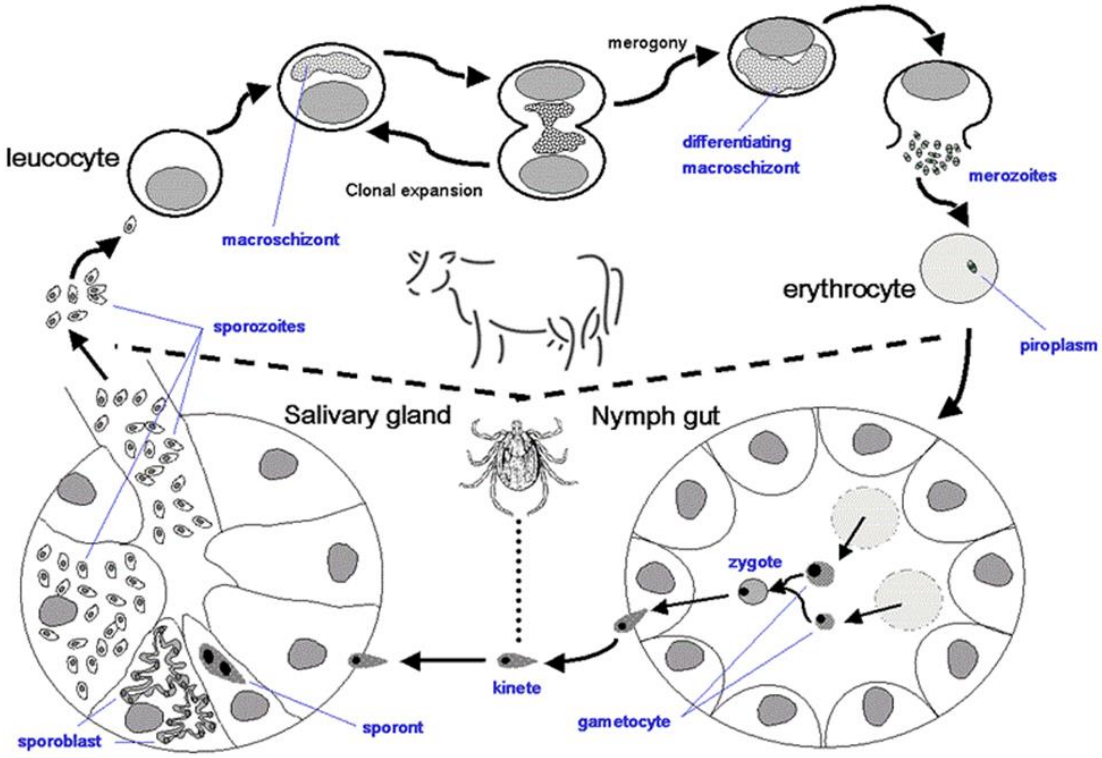

Figure 1.6. Life cycle of *Theileria annulata* (Source: http://www.theileria.org/ahdw/background.htm)

1.4.3.3. Diagnosis

Acute theileriosis can be diagnosed by clinical signs, history of tick exposure and microscopic identification of parasites in thin-blood, lymph node and tissue-impression smears (Geysen, 2014; Mans et al., 2015; Lempereur et al., 2017). Both *T. annulata* and *T. parva* can be found either as schizonts in the leukocytes or piroplasms within the erythrocytes. Multi-nucleated intracellular and extracellular schizonts are visible in lymph node smears in acute cases while piroplasmic stage is predominantly observed in recovering or mild cases (Geysen, 2014). As discussed earlier, microscopy is less sensitive method for the detection of carrier animals; thereby, negative results do not overrule the presence of latent infections (Mans et al., 2015).

Serological assays such as CFT, IFAT and ELISA can be used for the diagnosis of bovine theileriosis (Geysen, 2014; Mans et al., 2015). The IFAT has been the gold standard test for serological detection for a long time, but it lacks specificity in infections with multiple *Theileria* species. Although several attempts were made to develop ELISA using *T. annulata* antigens such as merozoites surface 1 antigen (Tams1), but to date, no ELISA kit is commercially available (Gubbels et al., 2000; Seitzer et al., 2007; Renneker et al., 2008; Geysen, 2014; Al-Hosary et al., 2015).

Molecular methods offer the most sensitive and specific means for the detection of *Theileria* spp. These methods include cPCR, qPCR, nPCR, blotting and LAMP assays (Mans et al., 2015; AL-Hosary et al., 2016; Gomes et al., 2017). Recent deep amplicon sequencing methods have revolutionised the diagnosis of haemoparasites and are being used worldwide with high sensitivity and specificity (Mans et al., 2016; Brinkmann et al., 2019; Chaudhry et al., 2019; Glidden et al., 2019).

1.4.3.4. Control methods

Control of bovine theileriosis is comparatively more challenging partly because there is no evidence of passive immunity and age-related resistance in calves reported to occur in other main TBPs (Cunningham et al., 1989; Toye et al., 2013; Morrison, 2015). Immunoprophylaxis, tick control and chemotherapy are the main control methods used worldwide (Morrison, 2015). Parasitised cell lines containing attenuated schizonts have been tested and used against both *T. parva* and *T. annulata* species since the 1960s in China, India, Israel, Iran, Spain, Turkey, northern Africa and central Asia (Pipano and

Tsur, 1966; Pipano, 1989; Geysen, 2014). However, due to risks of importing exotic pathogenic species, constraints associated with storage and transport of live vaccines and the requirement of a higher number of infected cells, the use of such vaccines is minimal (Morrison, 2015).

An alternative concept of 'infection and treatment' was introduced during the 1970s and was based on an infection with *T. parva* sporozoites followed by treatment with long-acting oxytetracycline (Radley et al., 1975a). Due to limited cross-protection against variants, Muguga cocktail vaccine was prepared containing a mixture of multiple isolates and is being widely used (Radley et al., 1975b; Di Giulio et al., 2009). Vector control relies on the control of ticks using acaricides which carry the disadvantage of related cost, resistance and environmental contamination as discussed earlier (Morrison, 2015).

Chemotherapeutic control relies on two main compounds, i.e., buparvaquone and halofuginone. However, these compounds are effective if used at an early stage of the infection. Furthermore, chemotherapeutic control sometimes results in carrier state due to partial clearance of theilerial infections (McHardy et al., 1985). Due to these challenges and other issues, including higher costs and emergence of resistant strains, the potential application of these chemotherapeutic agents has been limited (Mhadhbi et al., 2010; Sharifiyazdi et al., 2012). Future control options should include integrated tick control methods combined with the use of multivalent vaccines against major pathogenic *Theileria* species (Nene and Morrison, 2016).

1.5. Knowledge gaps in TTBDs of bovines from Pakistan

Even though several studies have investigated TTBDs in Pakistan, there is a paucity of information about the diversity, seasonal variation and risk factors of TTBPs. Reasons for these knowledge gaps include, but are not limited to, (i) use of less sensitive and/or conventional methods, (ii) use of questionnaire-based surveys, and (iii) convenience sampling in small geographical areas. Furthermore, there is no study available on the investigation of the vector potential of different tick species from Pakistan. Farmers can be a good source of important information about prevalence and risk factors for various animal-health related constraints including TTBDs. However, traditional methods of data collection (such as questionnaires) are not suitable for this purpose, particularly in resource-poor settings. Participatory epidemiology (PE) can provide a suitable alternative in such situations and has been successfully applied in animal-health investigations

(Catley, 2000; Catley et al., 2012). A brief account of PE methods and their potential uses is given in the following section.

1.6. Participatory epidemiology

1.6.1. Introduction

Participatory epidemiology is the systematic use of participatory methods and approaches to improve understanding of animal-health issues and devising control strategies (Catley et al., 2012). The word 'participatory' refers to the involvement of people associated with animal-keeping for devising prevention and control strategies (Catley et al., 2012). A more generalised definition of PE is that it offers vital roles to the stakeholders for designing and executing programmes targeted at public-health (Loewenson, 2004), animal-health, disease surveillance and research (Jost et al., 2007).

1.6.2. History and development of participatory epidemiology

The history of PE dates to early 1970s when it was found that the conventional methods of data collection were not well-suited for the rural development programmes (RDPs). There were six key drawbacks identified in RDPs which led to the development of PE (Chambers, 1983).

1. Spatial biases: ground applications of most of the RDPs were dictated by the availability of road access, transport, fuel and resting points during visits (Sennyonga, 1976; Moore, 1979). Contrary to this, most of the poor rural communities were lacking these facilities and therefore RDPs were concentrated in areas having such facilities.
2. Project biases: Most of the RDPs targeted those areas/villages which were presentable/accessible and therefore useful for showcasing to draw international attention.
3. Person biases: Presence of comparatively well-off/influential persons in certain villages attracted more RDPs in those villages. Male-dominated society also played an important role in ignoring female farmers. Furthermore, users of certain

facilities (such as school-going children, market-going farmers) had better chances of being involved in those projects.

4. Weather bias: Most of the international researchers tend to fly to in suitable seasons which are actually the seasons of least poverty (Longhurst and Payne, 1979; Chambers, 1982).
5. Diplomatic biases: The urban-based researchers lack direct contact with the farmers and are often misled by the field staff.
6. Professional biases: Professionally trained researchers tend to focus on progressing/educated farmers and thus ignore the poorest and illiterate farmers.

These drawbacks led to the development of various methods and approaches under rapid rural appraisal (RRA) in the 1970s (Chambers, 1990). Another key driver was the recognition of the fact that the local farmer's knowledge should be given importance. This knowledge, termed as indigenous technical knowledge (ITK), was about animals, crops and farming systems, which led to the increased involvement of rural people in development projects (Brokensha et al., 1980; McCall, 1987).

Veterinarians were already using two closely-related participatory methods (i.e. RRA and ITK) in the 1980s (Leyland, 1991) in Asia and Africa. During the late 1990s, there was an increase in the use of these methods, leading to the evolvement of PE (Catley, 2000; Alders and Spradbrow, 2001). Over the time, PE has evolved as an active surveillance system known as participatory disease searching in response to the needs of Global Rinderpest Eradication Program (Mariner and Roeder, 2003) in countries like Pakistan (Mariner et al., 2003; Hussain et al., 2005; Ali et al., 2006). It is now being applied globally for the disease surveillance (Thacker et al., 1988; Orenstein and Bernier, 1990; Mariner et al., 2003; World Organisation for Animal Health (OIE), 2007).

1.6.3. Participatory methods

Participatory epidemiology methods can be divided into three categories (i.e. informal interviews, visualisation methods and ranking and scoring). Table 1.8 provides an overview of the use of different methods of PE.

1.6.3.1. Informal interviews

Group and semi-structured interviews are two types of informal PE methods. Semi-structured interview (SSI) is a type of guided conversation in which the interviewer asks open-ended questions and later re-phrases questions as well as leads the conversation through follow-up of answers (Slim and Thomson, 1994; Pretty et al., 1995). Group interviews are like SSIs but involve a larger number of participants.

1.6.3.2. Visualisation methods

Visualisation methods were developed to overcome the issues faced while dealing with problems not expressible verbally or in written form as well as involving the illiterate informants. These include various diagrammatic tools applied on paper or on the ground using simple objects and enhance the involvement of group members. Seasonal calendars, participatory mapping, proportional piling and radar diagrams are important visualisation methods.

Participatory mapping is one of the most powerful tools of PE in which local communities draw their social structure, natural resources, livestock farms or village maps on paper or ground (Mascarenhas and Kumar, 1991; Ameri et al., 2009).

Seasonal calendars are widely used visualisation methods (Chambers, 1994; Catley et al., 2002b) to demonstrate the seasonal variation in rainfall (Catley et al., 2002a), humidity and temperature (Ameri et al., 2009), disease incidence (Konde, 1993; Hadrill and Yusuf, 1994), the occurrence of vectors (Catley and Aden, 1996), movements of livestock (Mearns et al., 1994; Elos et al., 1995), labour (Cooper and Gelezhamstin, 1994) and harvesting of livestock products (Mearns et al., 1994). Piles of counters are also used to get numeric estimates of variations on calendars (Barasa et al., 2008; Jost et al., 2010).

Proportional piling is a semi-quantitative visualisation method used to score the items against single criteria using simple countable objects like seeds, stones or beans (Mariner and Paskin, 2000; Ameri et al., 2009).

1.6.3.3. Ranking and scoring

The third main group of PE methods include simple, pairwise and matrix ranking or scoring. Like proportional piling, they also utilise piles of objects to rank or score various parameters. The results obtained from these methods are quantitative in nature and easy to analyse. Scoring methods are more sensitive than ranking methods because of their quantitative results (Catley et al., 2012).

1.6.4. Use of PE in animal-health related constraints

Participatory methods are now being widely used for the diagnosis, prioritisation, impact assessment, risk factors analysis and investigating seasonal variation of diseases and evaluation of disease-control programs as well as for the assessment of farmers' perceptions about diseases. For example, Catley and Aden (1996) used PE methods to investigate TTBDs in Somaliland. The study combined matrix scoring with seasonal calendar to quantify important problems related to tick infestation and their seasonal variation. In a similar study conducted in South Africa, attempts were made to assess farmer's knowledge about causes, symptoms, diagnosis and treatment of two important TBDs (anaplasmosis and babesiosis) using group interviews, SSIs, matrix scoring and mapping (Masika et al., 1997). In Pakistan, PE methods have been used in epidemiological studies on peste des petits ruminants (PPR), rinderpest, foot-and-mouth disease (FMD) and other livestock diseases (Ali et al., 2006; Anjum et al., 2006; Hussain et al., 2008; Zahur et al., 2008; Khan, 2010). Table 1.9 provides a list of selected animal-health issues which have been investigated using PE methods.

Table 1.8. Methods of participatory epidemiology and their potential uses

Method	Technique	Uses	References
Informal interviews	Semi-structured interviews	Used itself as well as in combination with visualisation and scoring methods	(Mariner and Roeder, 2003; Bagnol, 2007; Ahlers et al., 2009)
	Timeline	To collect information about history and timings of disease occurrence	(Admassu et al., 2005; Bagnol, 2007; Ahlers et al., 2009)
	Group interviews	To collect information from group of participants	
Visualisation methods	Participatory mapping	Mapping of livestock movement, natural resources (water, forests, grazing areas), vector distribution, roads	(Hadrill and Yusuf, 1994; Catley, 2004)
	Seasonal calendars	Seasonal variation in climatic conditions, vector population, disease incidence, fodder availability and farmer's livelihood	(Catley and Aden, 1996; Catley et al., 2002a; Bagnol, 2007; Ahlers et al., 2009)
	Proportional piling	Herd structure, disease variation by age groups, case fatality rates, variation in mortality rate due to vaccination	(Barasa et al., 2008; Rufael et al., 2008; Bekele and Akuma, 2009; Catley et al., 2009)
	Radar diagrams	Disease control strategies analysis	(Grace, 2003)
Ranking and scoring	Simple ranking	Disease control strategies analysis	(Grace, 2003)
	Simple scoring	To get priority list of important diseases	(Bedelian et al., 2007)
	Matrix ranking	To analyse different disease control options	(Catley et al., 2002a)
	Matrix scoring	To assess the local knowledge, to compare the farmers' diagnosis with veterinarians, local knowledge of disease vectors and evaluation of veterinary services	(Catley and Aden, 1996; Catley and Mohammed, 1996b; Admassu et al., 2005; Catley, 2006)
	Before-and-after scoring	To determine the impact of veterinary services on livelihood	(Admassu et al., 2005)

Table 1.9. Uses of participatory epidemiology in selected animal-health investigations

Diseases/Topics	Participatory Epidemiology Methods	Target group/ Species	References
TTBDs	Matrix Scoring, Seasonal calendar	Livestock	(Catley and Aden, 1996)
Several	Livestock disease scoring	Livestock	(Catley and Mohammed, 1996a)
Babesiosis Anaplasmosis	Matrix ranking, Diagramming, Group interviews, Semi-structured interviews	Cattle	(Masika et al., 1997)
Rinderpest	Map making, Historical point referencing, Group discussions	Cattle	(Jost et al., 1998)
Chronic wasting disease	Semi structure interview, Matrix scoring	Cattle	(Catley et al., 2001)
Trypanosomiasis	Secondary data and interviews, Matrix scoring, Proportional piling, Seasonal calendar	Cattle	(Catley et al., 2002a)
Several diseases	Informant group discussions, Seasonal calendar	Cattle	(Catley et al., 2002b)
Rinderpest	Group discussions, Semi structured interviews, Seasonal calendar	Cattle	(Mariner and Roeder, 2003)
FMD	Matrix scoring, Proportional piling	Livestock	(Catley et al., 2004)
Contagious bovine pleuropneumonia	Participatory disease searches, Proportional piling, Seasonal calendar	Cattle	(Mekonnen, 2004)
FMD	Interviews, Proportional piling, Matrix scoring, Participatory mapping, Seasonal calendar	Cattle	(Admassu, 2005)
Several	Semi-structured interviews, Disease impact scoring, Matrix scoring	Several	(Admassu et al., 2005)
Several	Not specified	Cattle	(Allport et al., 2005)
Several	Proportional piling, Seasonal calendar, Mapping, Key informants interview	Livestock	(Hussain et al., 2005)
Cattle mortality	Structured interviews	Cattle	(Makgatho et al., 2005)
Rinderpest	Proportional piling	Cattle	(Mariner et al., 2005b)
Rinderpest	Participatory disease searching and surveillance	Several	(Mariner et al., 2005a)
Several	Semi-structured interviews, Pairwise comparison, Matrix scoring, Seasonal calendar, Trend lines, Proportional piling	Camel	(Mochabo et al., 2005)

Several	Direct observation, Key-informant interviews, Mapping, Matrix scoring, Seasonal calendar, Proportional piling	Livestock	(Ali et al., 2006)
FMD	Key-informant interviews, Mapping, Seasonal calendar, Proportional piling	Cattle	(Anjum et al., 2006)
Comparison of veterinary knowledge b/w farmers and veterinarians	Matrix scoring	-	(Catley, 2006)
Malignant catarrhal fever	Focus group meetings, Key-informant meetings, Proportional piling, Matrix scoring, Priority disease scoring, Disease incidence, Disease impact scoring, Mobility mapping, Semi-structured questions	Cattle	(Bedelian et al., 2007)
Several	Brainstorming, Short lectures/talks, Focused and plenary, group discussions, Farm visits	Cattle	(Nkya et al., 2007)
Several	Social mapping, Semi-structured interview, Activity profiles, Seasonal calendar, Pie charts, Mobility diagram, Matrix ranking, Preference ranking & scoring, System analysis diagram, Focus group discussion	Cattle	(Shamsuddin et al., 2007)
Mastitis	Farmer group meetings, Participatory impact assessment	Cattle	(Vaarst et al., 2007)
FMD	Informal interviews, Proportional piling	Cattle	(Barasa et al., 2008)
PPR	Participatory disease surveillance	Sheep Goats	(Hussain et al., 2008)
Contagious caprine pleuropneumonia	Group interviews, Proportional piling, Matrix scoring	Goats	(Mekuria et al., 2008)
Several	Focus group discussion, Key-informant interviews, Proportional piling, Participatory mapping, Relative incidence scoring, Seasonal calendars, Probing	Livestock	(Bett et al., 2009)
Bovine dermatophilosis	Key informant meetings, Disease ranking	Cattle	(Chatikobo et al., 2009)
Several	Group discussions, Key-informant meetings, Seasonal calendars, Matrix scoring	Livestock	(Khan, 2010)
Anthelmintic resistance	Interviews, Focus group discussions, Field visits	Goats	(Saeed et al., 2010)

FMD	Key-informant meetings, Pair-wise ranking, Matrix scoring, Proportional piling	Cattle	(Shiferaw et al., 2010)
Low reproductive performance	Focus group discussions	Cattle	(Nqeno et al., 2011)
Ticks	Interviews, Focus group discussion	Cattle	(Adane et al., 2012)
FMD	Focus group discussions, Key-informant meetings, Pairwise comparison and ranking, Disease impact matrix (DIM) scoring, Proportional piling	Livestock	(Bellet et al., 2012)
Several	Semi-structured interview, Simple ranking, Proportional pilling, Pairwise ranking, Matrix scoring, Seasonal calendar, Mapping, Transect walk	Several	(Ndahi et al., 2012)
Several	Community meetings, Interviews	Cattle	(Hannah et al., 2012a)
Avian Influenza	Semi-structured interviews, Key informant interviews	Poultry	(Hannah et al., 2012b)
Rinderpest		Cattle	
Several	Focus group interviews, Key informant interviews	Livestock	(Malak et al., 2012)
	Transect walks, Natural resource use maps, Seasonal calendars,	Cattle	(Bardosh et al., 2013)
Trypanosomiasis	Focus groups, Interviews, Direct observations, Disease scoring, Household questionnaire		
Several	Focus group interviews, Key informant interviews, Proportional piling, Belt Transects, Brainstorming	Cattle	(Chatikobo et al., 2013)
FMD	Focus group discussion, Semi-structured interviews, Simple scoring, Pair-wise ranking, Matrix scoring, Proportional piling, Seasonal calendar	Cattle	(Jibat et al., 2013)
Trypanosomiasis	Structured interviews, Focus group discussions	Cattle	(Majekodunmi et al., 2013)
Rabies	Ranking, Scoring, Proportion piling, Seasonality Calendar, Open discussions	Livestock	(Okell et al., 2013)
Several	Listing, Pairwise ranking, Proportional piling, Disease incidence scoring, Disease impact matrix scoring, Probing	Cattle	(Onono et al., 2013)
Several	Pair-wise ranking, Matrix scoring, Semi-structured interviews	Livestock	(Bardsley and Thrusfield, 2014)
Several	Proportional piling	Livestock	(Catley et al., 2014)

Contagious bovine pleuropneumonia	Pair-wise ranking, Semi-structured interviews	Cattle	(Kairu-Wanyoike et al., 2014)
Several	Semi-structured interviews, Focus group discussions	Livestock	(Limon et al., 2014)
FMD	Focus group discussions, Semi-structured interviews	Cattle	(Nampanya et al., 2014)
Rift valley fever	Key informant interviews	Cattle	(Owange et al., 2014)
Several	Checklist, Semi-structured interview, Probing, Transect walk, Triangulations	Cattle	(Alhaji and Babalobi, 2015)
TTBDs	Semi-structured interviews, Proportional piling	Cattle	(Byaruhanga et al., 2015)
Anthrax	Focus groups, Semi-structured interviews, Structured questionnaires, Mapping, Proportional piling, Matrix and Ranking, Observations	Several	(Coffin et al., 2015)
Several	Field clinical observation, Matrix ranking and scoring	Livestock	(Molla and Delil, 2015)
Several	Semi-structured interviews, Focus group discussions, Simple ranking and scoring, Pairwise ranking, Proportional piling, Matrix ranking	Cattle	(Ayele et al., 2016)
Several	Group meeting, Disease impact scoring, Seasonal calendar, Triangulation	Cattle	(Elelu et al., 2016)
Several	Semi-structured interviews, Proportional piling	Livestock	(Ruíz et al., 2016)
TTBDs	Personal interviews, Focus group discussions	Cattle	(Sungirai et al., 2016)
Respiratory diseases	Focus group discussion, Semi-structured inter-views, Proportional piling, Matrix scoring	Camel	(Wako et al., 2016)
Delivery of animal health care services	Focus group discussions, Key informant interviews, Metaplan, Problem tree	Several	(Bugeza et al., 2017)

1.7. Conclusions, hypotheses and research aims

This literature review shows that the bovine population in Pakistan is known to be infested with ticks, particularly during the hot and humid months of the year, and the prevalence of TBDs is also high. However, there are major gaps in our knowledge of important aspects of TTBDs, including epidemiology, diversity, population genomics, transmission, treatment and control. The information available on the prevalence of TTBPs from Pakistan does not generally apply to different agro-ecological zones in the country due to the difference in the climatic conditions among these zones. Furthermore, the different compositions of local bovine populations, different husbandry practices and variations in the risk factors in different zones further complicate the situation and, therefore, need to be investigated in detail.

Most available epidemiological data on TTBDs have been acquired using conventional diagnostic methods, which are usually significantly less sensitive than molecular techniques. This has led to the underestimation of the prevalence of major bovine TTBPs in Pakistan. In some cases, molecular tools have been used to explore TBPs in tick populations rather than bovine populations, and there is no information available on the genetic variation in TBPs. Exploring this area could give useful insights for developing specific and sensitive diagnostic methods. Furthermore, to date, no vaccine has been registered for use against any of the major TTBPs. Hence, exploring key TTBPs will have important implications for the development of new vaccine candidates as well as trials using vaccines which are already available overseas. The information about the genetic composition of different TTBPs would be very useful in this regard.

Determining the endemic status of TTBDs is one of the most important pre-requisites for the control of such diseases, and surprisingly, this aspect has not yet been investigated in Pakistan. Pursuing this area would provide invaluable information on the prevalence of TBP infections in bovine populations, thereby yielding vital data to guide the relative risks of TBDs and developing tailored control strategies. Taken together, these considerations indicate that a comprehensive study should be designed in Pakistan to investigate (i) major bovine-health issues with a focus on TTBDs, (ii) the endemic status of TBDs, (iii) seasonal variation of ticks and associated risk factors and (iv) the prevalence of TBP infections in ticks and in bovines in different agro-ecological zones.

It is hypothesised that the knowledge of farmers in Pakistan will be important in designing future tick-control programs. It is proposed that the prevalence of TBPs is significantly higher than previously reported, mainly because insensitive methods have been used for testing. Moreover, it is hypothesised that there is significant variation in the prevalence of TTBDs among different agro-ecological zones in the country due to different climatic conditions and bovine breeds.

To test these hypotheses, the following research aims were set:

Aim 1: To investigate farmers and animal health professionals' perceptions about the major bovine health and production issues and TTBDs in different agro-ecological zones of Pakistan. (Chapter 2)

Aim 2: To assess the diversity of hard ticks infesting bovines in Pakistan. (Chapter 3)

Aim 3: To detect major pathogens and endosymbionts in bovine ticks. (Chapter 4)

1.7. References

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CHAPTER 2. A PARTICIPATORY INVESTIGATION OF BOVINE HEALTH AND PRODUCTION ISSUES IN PAKISTAN

2.1. Abstract

Systems to record the frequency of animal health events in Pakistan are limited. A participatory approach was used to address gaps in farmers' knowledge and understanding of bovine health and production issues in five agro-ecological zones (AEZs) of Pakistan. Participatory tools, including simple ranking, pairwise ranking, constraint impact scoring, and constraint profiling were used in group discussions with farmers and animal health professionals (AHPs) in six districts of two provinces, Punjab and Sindh. The results of the ranking activities showed that foot-and-mouth disease (FMD), clinical mastitis, ticks, haemorrhagic septicaemia, reproductive disorders, blackleg, and endoparasites were the most important bovine health and production constraints for small-scale dairy farmers. Constraint impact scoring showed that the participants perceived that: (1) milk production was severely affected by FMD and mastitis; (2) blackleg and parasitism led to poor growth rates and reduced meat production; (3) reproductive disorders and mastitis caused major economic losses (due to the high cost of treatment); and (4) blackleg and haemorrhagic septicaemia were the leading causes of mortality in cattle and buffaloes. Although there was strong agreement in responses and constraint impact scores between farmers and AHPs, farmers were more concerned about health issues that cause high mortalities, whereas AHPs emphasised the importance of disorders with a high economic impact. Despite socio-economic differences among AEZs, farmers' knowledge about bovine health and production constraints was similar. The findings from this study revealed that farmers had limited understanding of the risk factors and routes of transmission of various infectious diseases of bovines, which emphasises the need to develop and implement tailored extension programs in Pakistan to control contagious diseases of animals and to improve the profitability of small-scale dairy farmers.

2.2. Introduction

The farming of livestock animals represents a crucial source of food to almost a billion people worldwide (FAO, 2009, 2018b). Milk is a major commodity, obtained from ~ 123 million dairy farms globally, maintained predominantly in mixed crop-livestock and pastoral systems in Asia and Africa, respectively (Herrero et al., 2009; Herrero et al., 2012; Hemme, 2013). Estimates show that 68% of these farms are present in the Indian subcontinent, with > 80% of them being small-scale operations (Otte et al., 2012; Hemme, 2013). Animal health issues are a major constraint to milk production, particularly for small-scale dairy farmers, due to poor disease diagnosis and monitoring and a limited understanding of disease epidemiology (Devendra, 2001; Thomas et al., 2002). A similar situation exists in Pakistan, where the livestock sector plays a vital role in promoting socio-economic development in rural areas. Approximately eight million families, with a total of 30–35 million people in rural populations, are involved in livestock production activities, which contribute to 35–40% of their annual income (Rehman et al., 2017a; Government of Pakistan, 2019).

Pakistan is the third-largest milk-producing country in the world and has 47.8 million cattle (*Bos indicus* and *Bos taurus*) and 40 million water buffaloes (*Bubalus bubalis*) (Thomson et al., 1997; Hemme, 2018). Dairy animals are reared in four milk production systems: (1) rural subsistence smallholdings; (2) rural market-oriented smallholdings; (3) rural commercial dairy farms; and (4) peri-urban commercial dairy farms (12). Small-scale dairy farms (rural subsistence and rural market-oriented) with less than 10 animals per herd comprise > 90% of the total number of bovines in Pakistan and are predominantly in two provinces−Punjab and Sindh (Zia et al., 2011). The average milk yield per lactation on these smallholder farms is ~ 1,200 litres for buffaloes and 1,000 litres for cows, which is almost half of the national herd average for both species (Iqbal and Ahmad, 1999; Warriach et al., 2012).

The productivity of the Pakistani dairy sector is affected by constraints including nutrition, husbandry and health as well as limited access to vaccines and veterinary extension services (Wynn et al., 2006; Warriach et al., 2019). The major bovine diseases/syndromes reported from Pakistan include mastitis, foot-and-mouth disease (FMD), haemorrhagic septicaemia, blackleg (or black quarter) caused by *Clostridium chauvoei*, internal and external parasitic diseases and haemoglobinuria (Iqbal and Ahmad, 1999; Khan and Usmani, 2005; Ali et al.,

2006; Wynn et al., 2006; Khan et al., 2013; Ashfaq et al., 2014). In Pakistan, the annual losses due to animal diseases are reported to be US$ 200 million (Thomas et al., 2002). By comparison, in India, Birthal et al. (2006) recorded an annual loss of US$ 1.89 billion (138.3 billion Indian rupees) to farmers, ascribed to various animal diseases and an annual loss of US$ 430 million due to FMD alone (Devendra et al., 2000; Thomas et al., 2002). These losses could be reduced by adapting proper prevention and control measures against important diseases in developing countries. However, due to poor surveillance systems, there is little information on the incidence, distribution and dynamics of animal diseases in South Asian countries (Thomas et al., 2002). The productivity of dairy animals on small-scale farms can be sub-optimal due to poor husbandry practices and limited resources, such that these farms can pose a greater biosecurity risk for the spread of livestock and zoonotic diseases compared with commercial dairy farms (Hayes et al., 2017). Thus, it is important to investigate the knowledge base and practices of small-scale farmers regarding animal-health issues to prevent losses as well as to identify and mitigate biosecurity risks.

Undertaking studies in resource-poor communities is usually challenging because of their remote locations, limited infrastructure and challenges associated with data and sample collection as well as sample transport and storage (Goutard et al., 2015). Additionally, without a long-term relationship with target communities, a lack of confidence about governmental authorities can also be a challenge in such studies (Goutard et al., 2015). Despite these challenges, epidemiological investigations of livestock diseases should actively involve farmers to assess their knowledge and understanding of key issues and evaluate needs in local communities (Chenais and Fischer, 2018; Hamilton, 2018). Interviews, surveys and participatory epidemiological (PE) tools assist such studies.

The PE approach includes participatory rural appraisal (such as informal interviews, visualisation and mapping, scoring and ranking) combined with conventional epidemiological methods (Thrusfield, 2005). PE tools should reduce non-sampling errors recognised in questionnaire surveys, such as poor responses to questions and interviewer errors (Catley, 2006), and they are inexpensive and convenient to conduct with illiterate participants and provide validated results through triangulation of multiple methods (Mariner and Paskin, 2000; Bett et al., 2009). This approach is now being widely used in parts of Africa and Asia for disease surveillance, prioritisation, surveys, and control (Allepuz et al., 2017).

We elected to use a PE approach, to assess small-scale farmers' knowledge of bovine health and production (BH&P) issues compared with that of AHPs in five agro-ecological zones (AEZs) of Pakistan. The findings and conclusions from this study would address knowledge gaps and underpin future extension programs and biosecurity awareness campaigns for farmers, focused on increased farm productivity (Nampanya et al., 2010) as well as improved cooperation between farmers and AHPs.

2.3. Materials and methods

2.3.1. Study area

Based on the physiography, land use, soil type, and climate, Pakistan is divided into 10 AEZs (Pakistan Agricultural Research Council (PARC), 1980), and the distribution of bovines varies markedly across these zones (Agricultural Census Organization, 2006). Small-scale dairy farms are mostly located in five of the 10 AEZs: Indus delta, northern irrigated plains, arid (rain-fed), sandy desert, and southern irrigated plains (Agricultural Census Organization, 2006; Afzal, 2010). This study was conducted in four districts (Bahawalpur, Jhelum, Layyah, and Okara) of Punjab and the two districts (Sukkur and Thatta) of Sindh (Figure 2.1). These districts were selected based on operational convenience. However, each represented a different AEZ, except for the Arid zone, where two districts were selected (Jhelum and Layyah) to cover the diverse topography. The districts included are dominated by small-scale farmers, most of which are either landless or use rented land for agriculture, and mixed crop-livestock farming is usual. Men and women are mainly involved in livestock-related activities, with a varying degree of involvement of children. Many farmers with small-scale operations are resource-poor and, therefore, cannot afford to hire farm workers. Socio-economic status varies particularly between provinces with least developed communities in districts of Sindh province. Descriptive statistics of temperature and the population of humans, cattle and buffalo per study district are given in Table 2.1.

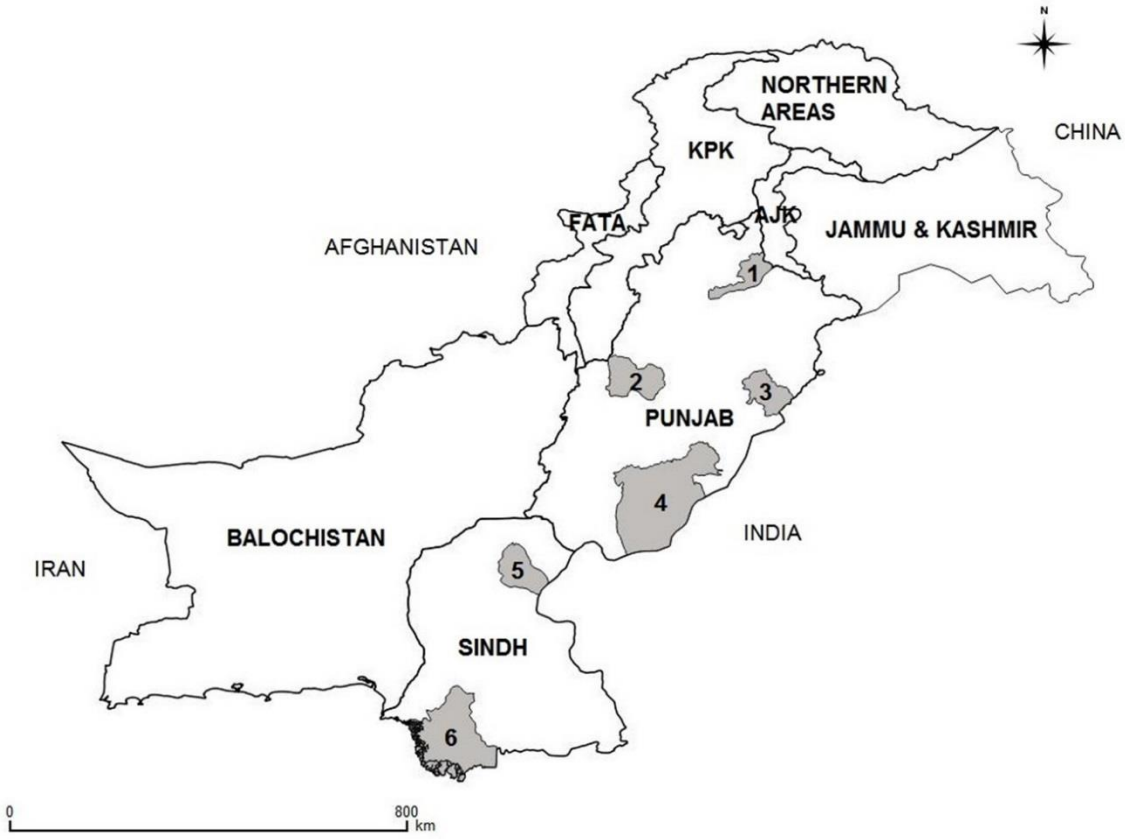

Figure 2.1. Map of Pakistan showing the locations of districts (grey-coloured areas) included in the study. The names of the districts are Jhelum (1), Layyah (2), Okara (3), Bahawalpur (4), Sukkur (5), and Thatta (6). The map was created using QGIS v. 2.18.15.

Table 2.1. Demographic properties of districts included in the study

Province	District	Agro-ecological zones	Temperature (˚C)[a] (Min-Max)		Population		
			Summer	Winter	Human (number)[b]	Bovine (million)[2] Cattle	Buffalo
Punjab	Bahawalpur	Sandy desert	39-46	1-7	3,668,106	0.79	0.81
	Jhelum[1]	Arid	38-45	0-6	1,222,650	0.24	0.19
	Layyah[1]	Arid	38-45	0-6	1,824,230	0.84	0.41
	Okara	Northern irrigated plain	39-46	1-6	3,039,139	0.47	1.18
Sindh	Sukkur	Southern irrigated plain	30-50	0-12	1,487,903	0.31	0.26
	Thatta	Indus delta	34-45	5-20	979,817	0.59	0.49

[1]*Two districts were selected from arid zone to cover geographic diversity of this zone.*
[2]*Estimated population in 2016 based on inter-census growth rate 1996 & 2006.*
a. (Afzal, 1997), b. (Pakistan Bureau of Statistics, 2017)

2.3.2. Structure of veterinary services in Pakistan

The provincial governments in Pakistan are responsible for providing veterinary services. Each province has its livestock department with various directorates offering services, including breed improvement, animal health, research, extension and disease surveillance (Afzal, 2009). Animal health professionals (AHP) include veterinarians and para-veterinary staff. There are veterinary hospitals, dispensaries and centres, which provide veterinary services to livestock farmers in most parts of the country. They are supported by a disease diagnostic laboratory in each district; however, most of such laboratories are under-resourced, in terms of well-trained qualified staff and facilities. The extension services are poor, and less than 40% farmers have access to any livestock extension program. A veterinarian heads each veterinary hospital and is supported by a veterinary assistant as well as a technician to undertake artificial insemination in bovines (Afzal, 2009; Wynn et al., 2017).

2.3.3. Study design

From September to November 2017, a cross-sectional study was conducted employing different PE methods. Following consultation with the Livestock Departments and the Dairy-Beef project, five villages were selected from each district. Eight to 10 small-scale dairy farmers (men) attended a facilitated group meeting (FGM) in each village; 30 FGMs were held in six districts. No women were involved in the FGMs, because of cultural and/or logistical issues. For AHPs, one FGM was conducted per district with each AHP group; 12 FGMs were held with AHPs (i.e., six with veterinary officers and six with veterinary assistants). No incentive was provided to farmers or AHPs for participation in any FGM.

2.3.4. Participatory epidemiology methods used

Four standard PE methods (simple ranking, pairwise ranking, constraint impact matrix scoring and constraint profiling) (Catley and Mohammed, 1996; Catley et al., 2002; Catley, 2006; Bardsley and Thrusfield, 2014; Ayele et al., 2016) were used to assess the knowledge of dairy farmers and AHPs on the major constraints to BH&P. Simple and pairwise rankings were used as triangulation to validate the list of top constraints in all FGMs. Constraint

impact matrix scoring was used to assess the impact of identified constraints on key health and production indicators. Constraint profiling informed about participants' knowledge about epidemiological aspects of the constraints. All FGMs were conducted in local languages.

The core research team consisted of two veterinarians and one veterinary assistant. One of the veterinarians acted as a facilitator, while the other recorded information on a board displayed at an appropriate distance from the participants. The veterinary assistant helped to organise FGMs and liaise with farmers. The FGMs were not voice-recorded to avoid any biases, because participants are usually reluctant to speak when they are audio and/or video-recorded, despite them being assured of privacy and confidentiality. The checklist of topics and PE techniques were pre-tested before the FGMs in a pilot study and the data were excluded from the final study. For each method, cross-checking and probing were carried out to: (1) validate the information, (2) ensure that the participants understood different items to be scored or ranked, and (3) ensure that enough information was gathered on each topic. During each activity, open-ended questions were asked to cross-examine the responses. However, utmost care was taken not to lead the participants to a specific health or production constraint. In each FGM, the participants were given time to discuss. If there was confusion about an issue or irrelevant discussion begun, the facilitator intervened through open-ended questions to guide the discussion, without forcing a consensus. The ranking, scoring and other aspects were recorded on charts. The same set of questions was used in all FGMs. Following each PE activity, the interview team met and reviewed the notes of each discussion to ensure that they were recorded objectively.

2.3.4.1. Simple ranking

Following a brief introduction of the team members and the objective of the study, participants were asked to name the important BH&P constraints causing high losses. In cases where the participants mentioned syndromes/symptoms as a constraint, probing, open-ended questions were asked to identify the local name of the constraint, but care was taken not to guide them to a particular BH&P constraint name. When the constraint name was not identified, further information was collected about the constraint until a proper diagnosis was reached. The constraint names were rechecked with the local veterinarian or para-veterinary staff at the end of the FGM. Once the constraints list was obtained, participants were asked

to rank each constraint according to its perceived importance (i.e., caused losses). This activity produced a list of constraints (mean: 9, range: 6–12) based on their perceived importance (ranked from 1 to 12, where 1 = most important and 12 = least important).

2.3.4.2. Pairwise ranking and comparison of constraints to bovine health and production

Pairwise rankings were carried out by comparing each constraint (already identified during simple ranking) individually with all other constraints. The simple ranking list of BH&P constraints was read aloud, and participants were asked again if they wanted to make any changes to the ranking order. Once re-validated, the top five constraints were taken from the list for pairwise ranking. The recorder sketched a 5 × 5 grid on a chart. The facilitator named the first constraint and asked participants to compare it with each of the remaining constraints, with supporting questions: (1) "Which of these two constraints is more important?"; (2) "Why is it more important?"; and (3) "Describe any differences/similarities between the two constraints." The recorder documented the constraint that was indicated as most important by participants. The total score (0-4) for each constraint in a group was recorded, with 0 being the least important and 4 the most important. This provided pairwise ranks for each constraint on a scale of 1 to 5, with 1 as the most important and 5 as the least important. The median scores for each constraint given by the 30 farmer groups and the 12 AHP groups were then used to establish an overall ranking.

2.3.4.3. Constraint impact matrix scoring

For each group discussion with farmers as well as AHPs, the five top-ranked BH&P constraints based on the pairwise ranking were scored against clinical and production-related indicators. These indicators were obtained during the pairwise comparisons in the pilot activity and included the impact on milk, meat, cost of treatment, morbidity and mortality. Indicators were written along the y-axis of a two-dimensional grid and the constraints along the x-axis. The facilitator asked the participants to distribute and/or assign a total of 10 small sticky dots to the five indicators for each of the constraints. Indicators and constraints were stated in the local language and explained to the participants when required. Participants were given time to develop consensus for voting, and once all the participants agreed on the

number of dots for each indicator, the sticky dots were pasted on the chart in corresponding boxes. The sticky dots were counted in each of the corresponding boxes for each indicator giving the impact score. The scoring procedure was repeated until all the indicators had been scored against each constraint.

2.3.4.4. Constraint profiling

This activity was conducted to assess the participants' knowledge about the identified BH&P constraints. The recorder wrote the first constraint on a chart, and the facilitator asked open-ended questions about the descriptors of a constraint, including local name(s), cause, clinical signs, risk factors, the season of occurrence, susceptible age and animal species involved, treatment and vaccine availability/schedule. Responses were recorded in the corresponding boxes, and the same steps were repeated for all the constraints in order of their pairwise ranks. Notes from the discussion were recorded separately.

2.3.5. Data analyses

Data from all FGMs were entered into a spreadsheet (Microsoft Excel 2016). Kendall's coefficient of concordance (W) was used to quantify the level of agreement for pairwise rankings of BH&P constraints among farmers and AHPs at the district as well as provincial levels. An agreement was assessed as weak, moderate and strong for values of W under 0.26, between 0.26 and 0.38 and greater than 0.38, respectively (Seigel and Castellan, 1998). To determine the overall association between constraint impact score (as the outcome variable) and the health and production indicators (as an explanatory variable), we developed a fixed-effects proportional odds (ordinal) logistic regression model. The proportional odds logistic regression models were estimated using the contributed R packages (Seigel and Castellan, 1998; R Core Team, 2017) including ordinal (Christensen, 2019), RVAideMemoire (Hervé, 2018), emmeans (Lenth, 2018), and lattice (Sarkar, 2008). See Appendix, Supplementary File S1: Data S2.1 (R code), Data S2.2 (Farmer FGM data), and Data S2.3 (AHP FGM data) used for the analyses.

2.3.6. Ethics approval

This study was approved by the Veterinary and Agricultural Sciences Human Ethics Advisory Group, The University of Melbourne (Ethics ID: 1748953). All activities involving human participants followed the ethical standards of protecting the rights and welfare of participants. Data were collected after the participants gave consent.

2.4. Results

2.4.1. Demographics of participants

The total number of participants included in this study was 277 (range: 8–10) and 83 (range: 5–8) in farmer and AHP FGMs, respectively. The farmers were adult men and mostly without any formal education; however, no data were recorded on the age and education of farmers. The AHPs included adult men (n = 79) and women (n = 4).

2.4.2. Simple ranking

A simple ranking of BH&P constraints showed that FMD, mastitis, ticks, haemorrhagic septicaemia, reproductive disorders, blackleg, and internal parasites were perceived to be important constraints by both farmers and AHPs (Figure 2.2). Farmers ranked FMD, mastitis, haemorrhagic septicaemia, reproductive disorders and ticks as major constraints in all AEZs. Similarly, redwater was also important in all AEZs, except the southern irrigated zone. Overall, farmers ranked haemorrhagic septicaemia as a major constraint due to higher mortality associated with this disease, particularly in Southern irrigated plain and Indus delta zones (Figure 2.2A). FMD was ranked among the top-five constraints due to its relatively high and unpredictable incidence and recurrent outbreaks. However, it was mostly ranked 2nd or 3rd due to associated low mortality and self-recovery. Mastitis was considered as one of the leading causes of low milk yield, high treatment costs and poor prognosis as well as the reduced value of the animal. Tick infestation was listed as one of the important issues due to its negative impact on the growth and production of animals, particularly during the summer season. Similarly, farmers cited blackleg as the cause of high mortality in the arid, sandy

desert and southern irrigated zones. Reproductive disorders including anoestrus, prolapse and repeat breeding were also considered as economically important constraints by the farmers (Figure 2.2A).

AHPs ranked mastitis, FMD, internal parasites, haemorrhagic septicaemia, blood parasites, reproductive disorders, ticks and blackleg among the top BH&P constraints (Figure 2.2B). They believed that mastitis was the most important health issue due to the low treatment success rate, leading to decreased milk production and reduced value of the animals. Despite mass vaccination campaigns against FMD by the provincial livestock departments, this disease was considered as the second major bovine health issue by AHPs. Internal parasites, mainly liver fluke, were also of major concern due to their negative impact on an animal's body condition and milk yield. Haemorrhagic septicaemia was considered a major health issue in Sindh province, and blackleg outbreaks were reported in arid and sandy desert zones of the Punjab province. AHPs also ranked ticks and tick-borne pathogens (i.e., *Anaplasma* spp., *Babesia* spp., and *Theileria* spp.) among major bovine health issues (Figure 2.2B). In contrast to farmers, AHPs mentioned that blood parasites were important in the northern irrigated, arid, and Indus delta zones.

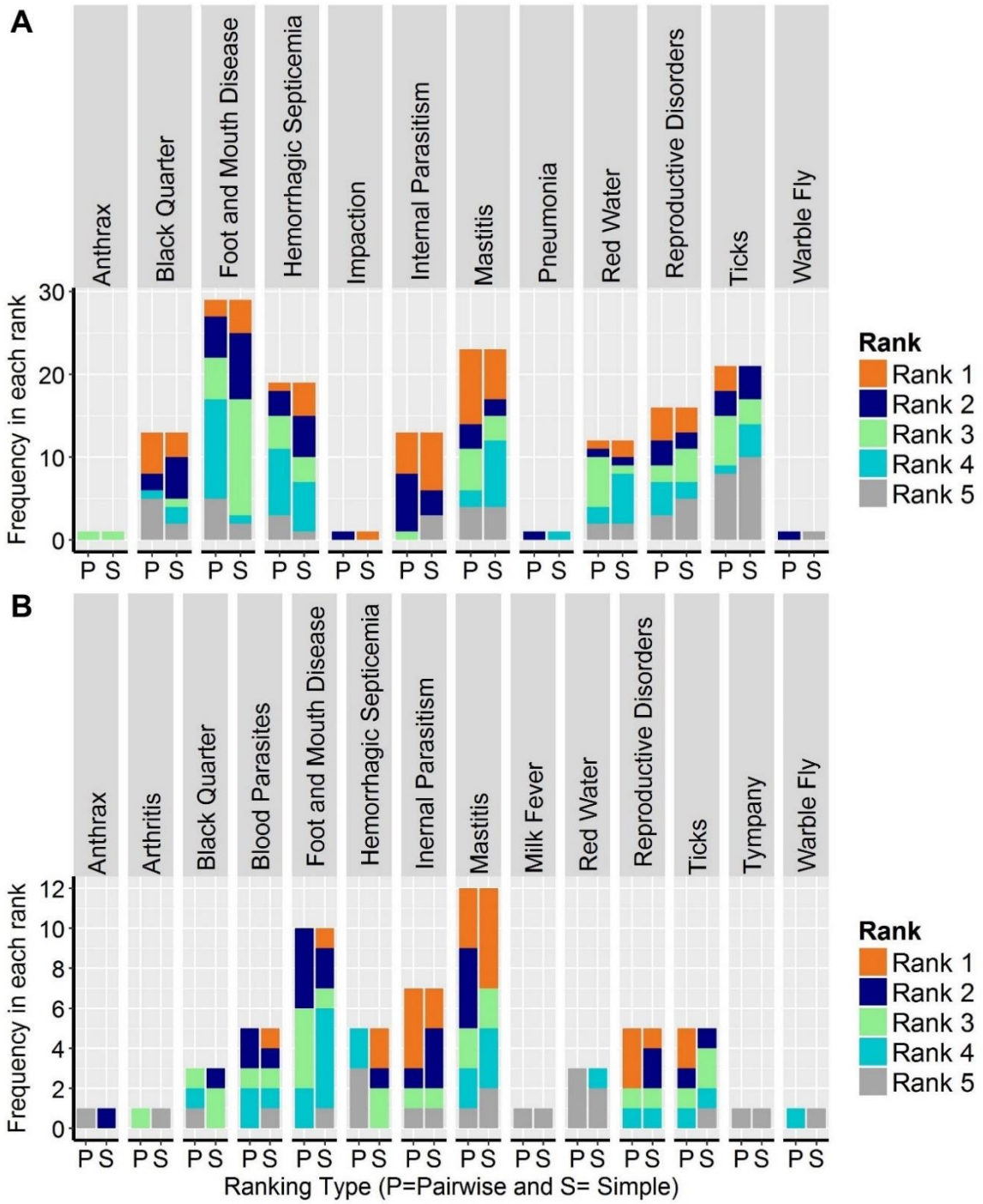

Figure 2.2. Grouped stacked bar plots of simple and pairwise rankings of major bovine health and production constraints by farmers (A) and animal health professionals (B).

2.4.3. Pairwise ranking

The pairwise ranking results showed that farmers ranked blackleg high when compared with other constraints due to higher mortality rates associated with it (Figure 2.2A). Mastitis was ranked higher due to low milk yield, the high cost of treatment and the poor prognosis. Similarly, AHPs considered the economic impact and prevalence rates of various constraints while comparing and ranking BH&P constraints. This activity resulted in changes in the ranking of all major issues; however, the overall ranking remained the same among both farmers and AHP (compare simple and pairwise rankings given in both Figures 2.2A, B). Kendall's coefficient of concordance showed strong agreement for pairwise ranks of BH&P constraints among farmers from all districts (W = 0.625, P = 0.000), as well as districts in Punjab (W = 0.718, P = 0.000) and in Sindh (W = 0.783, P = 0.045). The data from AHP FGMs from all districts also showed strong agreement (W = 0.915, P = 0.033).

2.4.4. Constraint impact matrix scoring

This method was used to compare the impact of major BH&P constraints on each of the five identified indicators on the livelihood of small-scale dairy farmers. Ordinal logistic regression of impact scores from both farmers and AHPs indicated that milk production was perceived to be severely affected by mastitis and FMD (Figure 2.3A). Farmers perceived that mastitis affecting one udder quarter could lead to an almost 25% reduction in milk yield and would also decrease the value of an animal, and FMD outbreaks impacted at least one complete lactation in a herd. Farmers thought that blackleg was the main issue for the low quality and yield of meat, while internal parasites, ticks, and redwater caused weight loss and reduced growth rate (Figure 2.3B). Furthermore, farmers believed that ticks suck blood while internal parasites utilise animal's intestinal food, thereby leading to weight loss (Figure 2.3B).

The cost was the third indicator for assessing the impact of BH&P constraints and the participants were asked about the economic impact (i.e., loss of income) and the expenses of treating a condition/constraint. Mastitis, redwater and reproductive disorders were considered as expensive constraints by both farmers and AHPs, whereas AHPs also included blood parasites in the list (Figure 2.4A). Both farmers and AHPs thought that mastitis

required costly and prolonged therapy with little success, while reproductive disorders (anoestrus in buffaloes and repeat breeding in cattle) caused losses to the small-scale dairy farmers in the form of increased calving interval, leading to higher costs involved in the feeding of dry animals. The fourth indicator was morbidity associated with a constraint/condition, and both groups identified that FMD, internal parasites, reproductive disorders (repeat breeding) and ticks had high morbidity (Figure 2.4B). According to farmers, FMD outbreaks occur biannually, and tick prevalence was usually higher during the summer season while internal parasites and reproductive disorder were prevalent throughout the year.

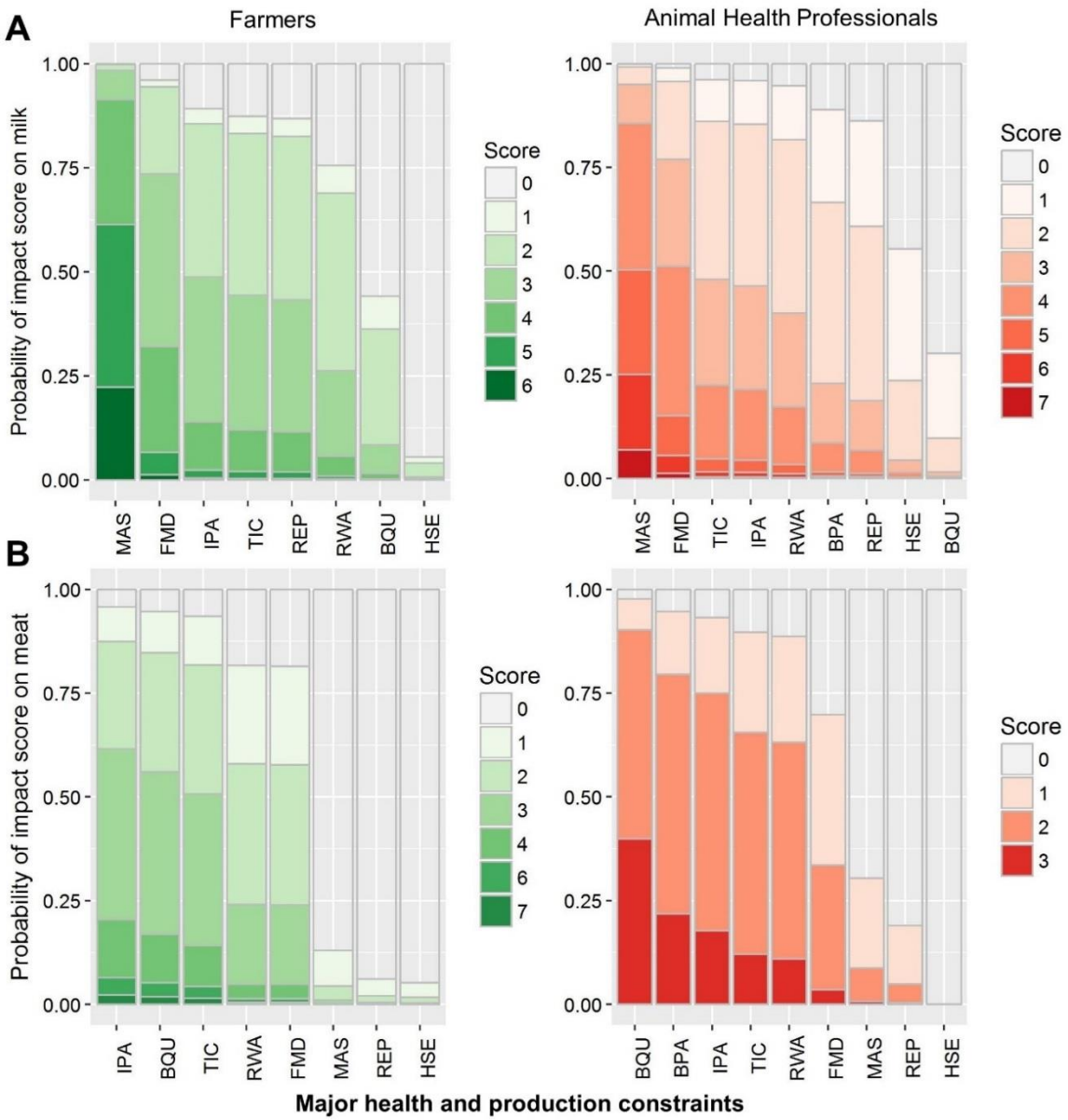

Figure 2.3. Grouped stacked bar plot of logistic regression of probability impact score of bovine health and production constraints on milk (A) and meat (B) production in cattle and buffaloes perceived by small-scale dairy farmers and animal health professionals in Pakistan. Major bovine health and production constraints include blood parasites (BPA), blackleg (BQU), foot-and-mouth disease (FMD), haemorrhagic septicaemia (HSE), internal parasites (IPA), mastitis (MAS), Redwater (RWA), reproductive disorders (REP), and ticks (TIC).

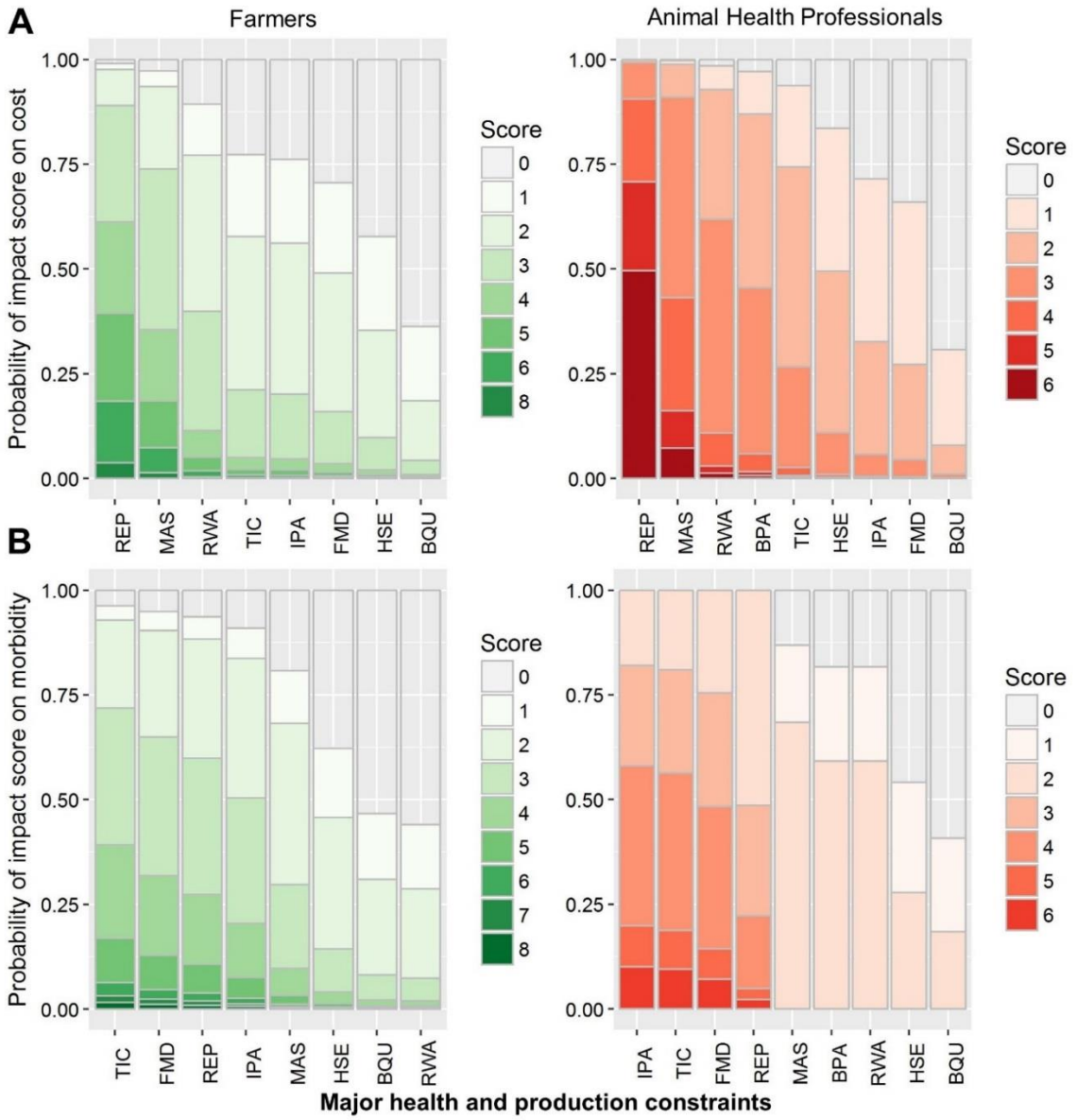

Figure 2.4. Grouped stacked bar plot of logistic regression of probability impact score on the cost of production (A) and morbidity caused by health and production constraints (B) in cattle and buffaloes perceived by small-scale dairy farmers and animal health professionals in Pakistan. Major bovine health and production constraints include blood parasites (BPA), blackleg (BQU), foot-and-mouth disease (FMD), haemorrhagic septicaemia (HSE), internal parasites (IPA), mastitis (MAS), Redwater (RWA), reproductive disorders (REP), and ticks (TIC).

Both farmers and AHPs commented that blackleg and haemorrhagic septicaemia were responsible for most mortalities (Figure 2.5). Further probing showed that, although the prevalence was much lower for blackleg, it was still considered as a major life-threatening issue for animals, due to its high case-fatality rate. Likewise, even though haemorrhagic septicaemia had been controlled in most of the areas through the extensive use of vaccination and antibiotics, it was still a cause of high mortality. Farmers also mentioned redwater as a cause of high mortality, particularly in buffaloes. Further questions determined that both post-parturient haemoglobinuria as well as haemoglobinuria caused by babesiosis, were classified as redwater by farmers as well as AHPs.

2.4.5. Constraint profiling

This method was used to assess the participants' knowledge of important health and production issues, their seasonal occurrence and the relevant epidemiological aspects (Table 2.2). In Punjab province, farmers were able to describe key clinical signs of BH&P constraints while in Sindh province, farmers provided limited information on this aspect (such as fever in FMD, difficult breathing in haemorrhagic septicaemia and reduced milk in mastitis). Overall, according to farmers, ethnoveterinary medicines were always the first line of treatment. In case of failure, they themselves used allopathic drugs, following the advice provided by AHPs. Farmers perceived that their animals got infected with FMD after either being in direct contact or close to infected animals, although their knowledge about risk factors of FMD was limited.

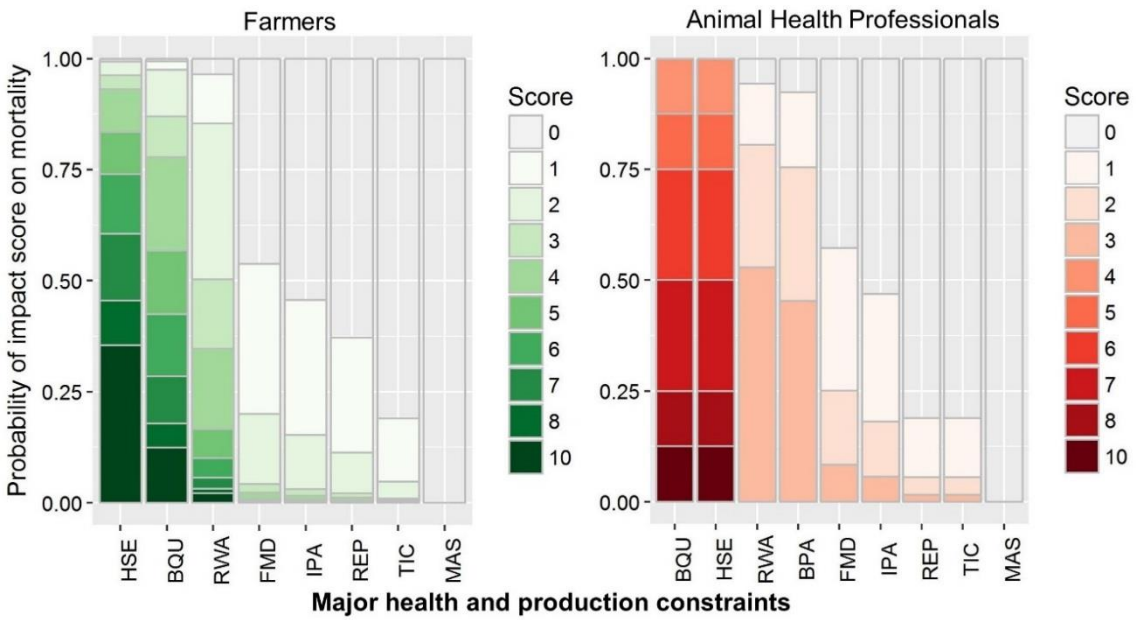

Figure 2.5. Grouped stacked bar plot of logistic regression of probability impact score on mortality caused by health and production constraints in cattle and buffaloes perceived by small-scale dairy farmers and animal health professionals in Pakistan. Major bovine health and production constraints include blood parasites (BPA), blackleg (BQU), foot-and-mouth disease (FMD), haemorrhagic septicaemia (HSE), internal parasites (IPA), mastitis (MAS), Redwater (RWA), reproductive disorders (REP), and ticks (TIC).

Table 2.2. Description of local names and clinical signs of major bovine health and production constraints described by farmers and animal health professionals

Constraint	Local name(s)	Clinical signs	Season of occurrence
Anoestrus	Wohry Nathi	Anoestrus and frequent vaginal discharge	Year round
Anthrax	Kaary Wa	Sudden death and oozing of blood from natural orifices	Year round
Black quarter	Chaurry Maar, Garri, Goli-Maar, Karo Wah Tangwari, Tangwara	Discoloration of meat (black), crepitation sound from rear leg muscles, fever, a hot area around swollen areas, lameness and sudden death	Apr-Aug
Foot-and-mouth disease	Munh Khur, Samarro, Muharro, Bhagiyo	Anorexia, drooling of saliva, fever, high mortality in calves, lameness, loss of production and vesicles on feet and in mouth	Feb-Apr, Sep-Nov
Haemorrhagic septicaemia	Gal Ghotu, Ghand, Ghogho, Ghanday	Coughing, difficulty in breathing, fever, swelling of the neck region and sudden death	Jul, Aug, Dec
Internal parasites	Keeray, Raig (Liver fluke)	Diarrhoea, hair loss, weakness, lacrimal discharge, loss of production, pendulous abdomen and submandibular edema	Year round
Mastitis	Angiyari, Changiyari, Munh Sarri, Phikriyo, Saaru,	Blood and clots in milk, fever, teat blockage/fibrosis, loss of production, pus in milk, salty taste, swollen udder/teats and ulcers on teats	Year round
Redwater (haemoglobinuria)	Ratt Mutra, Sarkan, Saro	Blood in urine, constipation, frequent defecation (in small amounts), fever, loss of appetite, a low temperature of hindquarters	Jan, Feb, Aug, Sep
Repeat breeding	Phiraal	Repeated heat signs, increased vaginal discharge and a very high or low body condition score	Year round
Ticks	Chichar, Katway	Anaemia, rough body skin, itching, skin lesions, ticks visible with naked eye and lassitude	Apr-Aug

Discussion on epidemiological aspects of mastitis revealed that infectious agents, unhygienic conditions and incorrect milking practices were the main contributing factors. For tick infestation, hot season, grazing, and lack of backyard poultry and water ponds were mentioned as the main risk factors. Tick control methods varied from region to region and mainly involved manual removal of ticks and burning, application of used engine oil, diesel, petrol and taramira oil (*Eruca sativa*) on animal's body besides periodical use of acaricides (such as avermectins, cypermethrin, trichlorfon). Haemorrhagic septicaemia was identified as a disease with the highest mortality rate in young animals (6–24 months) during the rainy season. Haemorrhagic septicaemia was mainly reported in both districts (Sukkur and Thatta) of Sindh due to the lack of vaccination. The main reproductive disorders were anoestrus, repeat breeding and prolapse both in cattle and buffaloes. Redwater (haemoglobinuria) was ascribed to parturition, phosphorus deficiency and tick-borne pathogens.

Constraint profiling activity was also repeated with AHP to cross-examine farmer knowledge, and a strong agreement was found in responses between both groups. However, understandably, AHPs had better veterinary science knowledge about all aspects (causes, risk factors, clinical signs, treatment) of BH&P constraints compared with the farmers. Within AHPs, the knowledge level was comparable, but veterinary officers had a sound background in veterinary medicine as compared to veterinary assistants who possessed a comparatively better understanding of local names of constraints.

2.5. Discussion

This study provides a detailed account of the important BH&P constraints that affect the livelihoods of small-scale dairy farmers in Pakistan. It also gives insights into the knowledge of farmers and AHPs about these constraints across different AEZs of the country. Previously, PE tools have been used mainly for animal health investigations in pastoral communities (Catley et al., 2001; Mariner and Roeder, 2003; Catley et al., 2004; Alhaji and Babalobi, 2015; Byaruhanga et al., 2015; Queenan et al., 2017). Pastoral communities are common in African countries whereas small-scale mixed crop-livestock farming is common in South Asian countries like Pakistan and India which have two-thirds of global dairy farms and contributing a quarter of global milk production (Hemme and Otte, 2010; Hemme, 2013).

The findings of this study are likely to reflect the situation in other provinces and neighbouring countries because of similar farming and education systems. Inter-group bias was minimised by using the same research team to conduct all FGMs; however, despite all efforts, some information might have been lost. As only male farmers were involved in FGMs, some of the important information from women and/or children might have been lost. Furthermore, no data were collected about farmers' age, economic status, education, and their level of involvement in livestock-related activities; hence, no conclusions can be drawn about differences resulting from these factors. As all participants were volunteers from diverse socio-economic backgrounds from different districts, the findings of this study should be interpreted with caution. Furthermore, the selection of villages was not random in this study due to limitations in accessing distant locations. It is envisaged that these challenges will be considered in the design of future studies.

Blackleg, FMD, haemorrhagic septicaemia, internal parasites, mastitis, ticks and reproductive disorders were the main BH&P constraints for small-scale dairy farmers. Ghaffar et al. (2007) and Khan (2010) used PE tools to investigate issues and infectious diseases of bovines in Punjab and found that FMD, haemorrhagic septicaemia, blackleg and mastitis were the major issues. Our results from the southern irrigated zone (Sukkur, canal-irrigated) also showed that FMD and haemorrhagic septicaemia were the most important infectious diseases of bovines. However, earlier studies did not focus on small-scale dairy farmers, and none of them investigated farmers' understanding of the constraints for the productivity of their animals, rather, their focus was on the surveillance of infectious or transboundary diseases of livestock. To the best of our knowledge, this is the first study that utilised four complementary PE techniques to investigate the knowledge of farmers and AHPs about BH&P constraints faced by small-scale dairy farmers in Pakistan.

We found that farmers prioritised constraints based on the mortality rate associated with the constraint/condition, whereas AHPs ranked constraints based on their economic importance. This finding is consistent with previous reports by Ali et al. (2006) and Hussain et al. (2005) from Pakistan and Chatikobo et al. (2013) from Zimbabwe. This difference of prioritising constraints could be because small-scale dairy farmers use bovine animals as their primary source of income and any bovine health constraint which leads to mortality in the herd would have impacted their income, thereby making them remember such constraints.

Another plausible cause could be due to the lack of record-keeping in small-scale dairy farming systems. Furthermore, it also means that farmers pay less attention to those health and production constraints which are subclinical and do not lead to mortality of animals. These constraints (e.g., ticks, tick-borne pathogens, internal parasites) could be crucial for improving the productivity of their animals. Future extension programs targeted at farmers should focus on educating them about endemic BH&P constraints which cause production losses round the year.

In Punjab province, farmers' description of important clinical signs of BH&P constraints matched with that given by AHPs. However, in Sindh province, farmers were unable to describe constraints/conditions clearly. This might be due to the lack of veterinary services, poor implementation of extension programs targeted at farmers, poor adoption rates of an existing extension program and lower education level of farmers, also identified by Ali et al. (2019). Given that most of the small-scale dairy farmers in Sindh belong to very low-income communities where the role of women in livestock rearing is usually higher, men might possess less information about constraints to livestock production (Batool et al., 2014). Hence, the difference in responses by farmers from Punjab and Sindh could be due to the different levels of involvement of men in livestock-related activities, but no data were collected to support this observation.

In Pakistan, the livestock extension programs targeted at livestock farmers are outdated and are mostly male-oriented (Burton et al., 2012). Previous studies have shown a higher involvement of women in day-to-day animal-related activities which varied from region to region and were indirectly governed by farmers' economic status (Shafiq, 2008; Batool et al., 2014; Wynn et al., 2017). Recently, Wynn et al. (2017) reported that the current extension services in Pakistan cover only 40% of small-scale farmers, a majority of which are men who have a minimal role in day-to-day livestock activities and thus lead to poor adoption of extension messages. This is supported by Warriach et al. (2019) who implemented the whole-family approach (by involving men, women and children) for the extension program in Pakistan and found higher adoption rates leading to better animal health and on-farm benefits. Therefore, a whole-family approach for extension services might be a better approach to educate small-scale dairy farmers about health and production constraints for their animals.

In this study, the small-scale dairy farmers used ethnoveterinary medicines as the first choice of treatment for their animals because of high cost and perceived inefficacy of allopathic drugs, and in some cases unavailability of veterinary services in remote areas. A similar situation exists in many developing countries where resource-poor farmers are unable to afford the costly allopathic drugs and therefore, choose ethnoveterinary medicines that are cheap, easily available and have been used over generations (Kudi, 2003). However, some infectious diseases (such as FMD and haemorrhagic septicaemia) cause epidemics and require the use of broad-spectrum antibiotics and vaccines for treatment and prevention (Kudi, 2003). Therefore, it is essential to develop integrated approaches to control BH&P constraints faced by small-scale dairy farmers where the ethnoveterinary medicines practices are validated and conserved, and allopathic drugs and vaccines are also appropriately prescribed by AHPs.

Farmers informed that the introduction of new animal and lack of vaccines were responsible for FMD and haemorrhagic septicaemia, respectively. Other causes identified by farmers were unhygienic conditions (mastitis), grazing practices (ticks), poor quality water (liver fluke), nutritional deficiencies (reproductive disorders), and calving (redwater) or unknown agents. Farmers were able to describe the seasonal occurrence of BH&P constraints. They were dependent upon government-funded vaccination schemes in Punjab whereas, in Sindh, such schemes are not common and small-scale dairy farmers in remote areas do not have access to such schemes or veterinary services. These results show that small-scale dairy farmers had a good understanding of the common BH&P constraints. However, our results are contrary to those of Arif et al. (2017) who found that most of the farmers did not know about brucellosis and its zoonotic potential. This difference could be due to limited knowledge of farmers about zoonoses and their importance versus important BH&P constraints which directly affect their livelihoods.

FMD is a highly contagious disease, and despite the use of local and imported vaccines, it is the most prevalent disease of bovines in Pakistan (Zahur et al., 2006; Jamal et al., 2010; Abubakar et al., 2015). However, farmers ranked FMD second to haemorrhagic septicaemia or blackleg because of low mortality rate, particularly in adult animals. Similar findings were reported by Bellet et al. (2012) who found that farmers ranked FMD second to haemorrhagic septicaemia as it caused lower mortality in Cambodia. Interestingly, in the Indus delta in

Sindh, FMD is considered as a fever-syndrome as well as a sign of good fortune for the animals, and farmers inoculate their animals with saliva or soil from hooves of infected animals. Although this practice leads to active immunisation, it may also lead to clinical disease due to pathogenic strains of the FMD virus. Another practice to control FMD in Punjab was keeping or burning a camel's bone in the herd as farmers believed that FMD did not affect camels. Although the dromedary camels are not susceptible to FMD and do not transmit it to other animals (Wernery and Kinne, 2012), there is no scientific evidence to support this myth of keeping or burning camel bones in the herd to prevent FMD. Ferrari et al. (2014) and Jemberu et al. (2014) estimated the economic impact of FMD in Pakistan and Ethiopia, respectively, and found that high economic losses occurring mainly due to loss of milk yield could be prevented by adopting timely appropriate preventive measures such as vaccination and biosecurity measures.

Currently, Pakistan is at stage-2 of FMD Progressive Control Pathway (PCP) which commenced in 2008, in collaboration with FAO and OIE (FAO, 2018a). The present study demonstrates that farmers usually lack knowledge about risk factors for transmission of FMD and engage in several risky practices. To advance to stage-3 of FMD PCP, an extensive farmer extension program should be implemented in the country, providing critical information on routes of transmission and risk factors. Additionally, designing and implementing an FMD simulation model would also be pivotal for controlling losses due to FMD in Pakistan and other endemic countries (Pomeroy et al., 2017).

In this study, both farmers and AHPs ranked mastitis as one of the major causes of losses on small-scale dairy farms in Pakistan where it is highly prevalent in dairy animals (Iqbal et al., 2004; Ali et al., 2008; Khan, 2010). Small-scale dairy farmers treat mastitis with ethnoveterinary medicines such as garlic and red chili, and they do not contact the AHPs until the chronic stage of mastitis develops due to the high cost of allopathic drugs.

Gastrointestinal parasitism was also ranked as a major cause of economic losses to small-scale dairy farmers across all AEZs. Farmers reported liver fluke (*Fasciola hepatica* and *F. gigantica*) infection within both AEZs of Sindh province. This could be due to the location of these areas along the river-bank, which provide a suitable environment for the intermediate host (snail) of liver flukes (Bhutto et al., 2012). In Sindh, small-scale dairy farmers have low income, and they cannot afford to deworm their animals for liver fluke. Like in Punjab,

scheduled deworming programs for livestock in Sindh can help to reduce production losses associated with gastrointestinal parasitism in dairy animals.

Farmers and AHPs considered tick infestation as an important BH&P constraint in all AEZs. The clinical signs of tick infestation mentioned were visibility of ticks with the naked eye, general weakness of animals and skin lesions caused by bites. Farmers believed that tick infestation was endemic throughout the year due to inefficacy of available acaricides and production losses were caused mainly due to the blood sucked by ticks. Previously, poor husbandry practices have been found to be associated with tick infestation on small-scale dairy farms (Sajid et al., 2009; Farooqi et al., 2017; Rehman et al., 2017b). Manual grooming is a widely practised method by small-scale farmers for tick control; however, it is time-consuming and bears a risk of the transmission of zoonotic diseases such as Crimean-Congo haemorrhagic fever. Farmers did not perceive tick-borne pathogens as a threat (in contrast to AHPs) to their animals. However, anaplasmosis, babesiosis and theileriosis are endemic bovine tick-borne diseases (TBD) in Pakistan (Jabbar et al., 2015). Recent investigations have demonstrated that bovine ticks carry a diverse array of pathogens, some of which can be zoonotic (Ghafar et al., 2020). Losses due to TBDs could be very high in Pakistan, particularly on resource-poor farms. Recently, Sungirai et al. (2016) investigated farmers' perceptions about TBDs in Zimbabwe and found that 67% of farmers were able to describe TBDs with signs. The high level of TBDs awareness resulted in farmers engaging in precautionary measures such as regular acaricidal dipping of their animals. Similarly, small-scale dairy farmers in Pakistan should be provided with information about the role of ticks as vectors and the adverse impact of ticks and TBDs on the health of their animals.

2.6. Conclusions

Despite the pivotal contribution of bovines to the food and livelihood of small-scale dairy farmers of Pakistan, the productivity of cattle and buffaloes is limited by health and production issues, including blackleg, FMD, haemorrhagic septicaemia, mastitis, ecto- and endo-parasites, and reproductive disorders. Farmers possess good knowledge about these constraints; however, they lack sufficient information about the risk factors about important BH&P constraints. There is no variation of constraints between/among different AEZs within the same province. In most cases, traditional (herbal) remedies are used to treat BH&P

constraints. Farmers prioritise constraints that cause high mortalities, whereas AHPs consider economic losses for ranking constraints. This study emphasises the need for simple extension programs using a whole-family approach by covering all critical aspects of BH&P constraints to prevent economic losses to small-scale dairy farmers.

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CHAPTER 3. EXPLORING THE PREVALENCE AND DIVERSITY OF BOVINE TICKS IN FIVE AGRO-ECOLOGICAL ZONES OF PAKISTAN USING PHENETIC AND GENETIC TOOLS

3.1. Abstract

Tick infestation is a leading cause of tick-worry and tick-borne diseases in livestock and associated economic losses in tropical and subtropical regions of the world. The cattle and buffalo populations in Pakistan are exposed to tick infestation throughout the year, but very little is known about the biology, diversity and distribution of tick species across different agro-ecological zones (AEZ) of the country. The present study aimed to investigate the prevalence (number of bovines infested with ticks out of the investigated population) and diversity of hard ticks infesting bovines in 30 villages located in five distinct AEZs (i.e. Arid, Indus delta, Northern irrigated plain, Sandy desert and Southern irrigated plain). We collected a total of 774 ticks (adult and nymphs) from cattle (n = 116) and water buffaloes (n = 88) on small-holder dairy farms (with < 10 bovids per establishment) from September to November 2017. The overall tick prevalence was 46.1% (cattle: 47.9%; buffaloes: 44%), which varied significantly from 22.2% in the Indus delta to 70.5% in the Sandy desert. Tick prevalence was slightly higher in female (46.5%) than male animals (45%), and higher in calves (i.e. ≤ 1 year of age) (55%) than in young animals (i.e. up to 3 years of age) (39%) and adults (48%). Five tick species – *Hyalomma* (*Hy*.) *anatolicum*, *Hy. hussaini*, *Hy. scupense*, *Rhipicephalus* (*Rh.*) *microplus* and *Rh. annulatus* - were identified morphologically and then genetically. Genetic identification, achieved using the sequences of two mitochondrial (cytochrome *c* oxidase subunit 1 and 16S) and one nuclear ribosomal (second internal transcribed spacer) regions, was consistent with the morphological findings. Phylogenetic analyses of the DNA sequence data sets showed that the five species of tick identified here were closely related to the same species or closely related species from within and outside of Pakistan. Of five presently recognised taxa within the *Rh. microplus* complex, two were identified herein, i.e., *Rh. microplus* clade C and *Rh. annulatus*. This investigation provides

the first genetic evidence of the occurrence of *Rh. annulatus* in Pakistan as well as *Hy. hussaini* and *Hy. scupense* in bovines specifically in the provinces of Sindh and Punjab, respectively. The present findings emphasise the importance of combining morphological and molecular approaches to study the diversity of ticks. Further longitudinal studies are required to establish seasonal variations in the prevalence and distribution of bovine ticks in different AEZs of Pakistan.

3.2. Introduction

Ticks (Acari: Ixodida) are obligate, haematophagous ectoparasites of terrestrial vertebrates worldwide. They can affect livestock, directly, by causing irritation, allergy and paralysis or, indirectly, by transmitting various pathogens, such as bacteria, viruses and protozoa (Uilenberg, 1992; Jongejan and Uilenberg, 2004; Anderson and Magnarelli, 2008; Sonenshine and Roe, 2014). It is estimated that 80% of the global cattle population is at risk of ticks and tick-borne diseases (TTBDs) (de Castro, 1997), resulting in annual global production losses of ~ USD 22 billion to 30 billion, with the majority of these losses occurring in developing countries (Lew-Tabor and Valle, 2016). Economic losses attributable to ticks in cattle are caused either by tick-worry, blood losses, damage to hides and udders, tick toxins, or through mortality or debility caused by tick-borne diseases (TBDs). These substantial losses adversely impact on the livelihood of poor-resource farmers due to reduced milk yields and live weight gains in bovines as well as damage to hides (Jongejan and Uilenberg, 2004).

To date, a number of studies has shown that > 80% of bovines in Pakistan are exposed to substantial tick infestations with species of *Hyalomma* and *Rhipicephalus* (Agricultural Census Organization, 2006; Sajid et al., 2008; Iqbal et al., 2013; Farooqi et al., 2017; Rehman et al., 2017) which transmit *Theileria annulata*, *Babesia* and/or *Anaplasma* spp. to cattle and buffalo (Jabbar et al., 2015; Karim et al., 2017). Recent epidemiological investigations of TTBDs have revealed that the ticks of bovines harbour a more diverse spectrum of pathogens than previously recognised in Pakistan (Karim et al., 2017; Rehman et al., 2019).

The accurate identification of ticks to species is central to understanding TTBDs and to support effective and sustainable control strategies for these diseases (Diarra et al., 2017). Currently, morphological (phenetic) identification of the adult stages of ticks is the main method used in Pakistan. However, species identification by morphological means can be challenging for engorged or damaged tick specimens and immature larval and nymphal stages, particularly in diagnostic laboratories with limited entomological expertise (Caporale et al., 1995; Walker et al., 2003). Furthermore, several tick species are morphologically very similar (e.g. *Hyalomma* (*Hy*.) *anatolicum* and *Hy*. *excavatum* and also members of the *Rhipicephalus* (*Rh*.) *microplus* complex), thereby often preventing accurate identification (Guglielmone et al., 2014; Roy et al., 2018).

DNA barcoding (Navajas and Fenton, 2000; Cruickshank, 2002) provides an alternative approach for tick identification. Genetic markers, including mitochondrial cytochrome *c* oxidase subunit I (*cox*1) and 16S ribosomal RNA genes and/or the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA have been used for this purpose (Black and Piesman, 1994; Zahler et al., 1997; Barker, 1998). However, no single molecular marker is ideal, such that a combination of nuclear and mitochondrial markers is advantageous (Cruickshank, 2002). For example, the sequence of *cox*1, has been used extensively to identify ticks, because of its apparently high evolutionary rate and its multi-copy nature relating to high PCR amplification efficiency (Hebert et al., 2003a; Hebert et al., 2003b). Similarly, mitochondrial 16S gene sequence data have been used widely to study the systematics of ticks at the family level (Black and Piesman, 1994; Lv et al., 2014b). Furthermore, ITS-2 sequence data have been used to identify and differentiate closely-related species of ticks as well as to resolve their complex phylogenies (e.g., (Zahler et al., 1997; Barker, 1998; Navajas and Fenton, 2000; Cruickshank, 2002; Yao et al., 2010; Lv et al., 2014a).

Despite the high prevalence of TTBDs and economic losses caused by them, very little is known about the genetic diversity of ixodid ticks in Pakistan. Few studies (Rehman et al., 2017; Sands et al., 2017; Roy et al., 2018; Ali et al., 2019; Zeb et al., 2019) have attempted to genetically characterise ticks from different parts of this country. However, these studies have some limitations, including the use of sequences for one or two genetic markers (*cox*1 and ITS-2) to identify small numbers (n = 19) of tick specimens (Rehman et al., 2017)

representing only one genus - *Rhipicephalus* (Roy et al., 2018; Ali et al., 2019) or *Hyalomma* (Sands et al., 2017) – or small geographical areas (Zeb et al., 2019). Importantly, no study has yet genetically characterised a range of tick taxa from different agro-ecological zones (AEZs) in Pakistan. Therefore, the present study was aimed at investigating bovine ticks across five AEZs of Pakistan using a combined morphological and molecular approach.

3.3. Materials and methods

3.3.1. Study design and sample collection

Based on climate, physiography, soil type and land use, Pakistan is divided into 10 AEZs: (i) Indus delta, (ii) Southern irrigated plain, (iii) Sandy desert, (iv) Northern irrigated plain, (v) Arid, (vi) Wet mountains, (vii) Northern mountains, (viii) Western dry mountains, (ix) Dry western plateau and (x) Sulaiman piedmont (Khan, 2004). The populations of cattle and buffaloes are mainly concentrated in irrigated, arid, desert and Indus delta zones of the country; thus, the present cross-sectional study included these five AEZs.

In the present study, the sample size of farms was calculated from a large population of bovines with the desired precision of 10% and a confidence interval (CI) of 95%. The calculated sample size of 97 farms was increased to 150 for uniform distribution across all AEZs (Cannon and Roe, 1982). In the first stage, one district was selected from each AEZ (except the arid zone, from which two districts were selected due to its diverse topography). From each district, five villages were randomly selected, and five farms were visited in each village. Assuming a tick prevalence of 50%, randomly selected half of the bovines on a farm were examined for tick infestation, resulting in a minimum of 1 and maximum of 8 animals per farm.

A total of 242 cattle (*Bos taurus* and *Bos indicus*) and 200 water buffaloes (*Bubalus bubalis*) were examined for tick infestation from September to November 2017 in 30 villages of the six districts in Punjab province, including Bahawalpur (Sandy desert), Okara (Northern irrigated plain), Jhelum and Layyah (Arid), and the districts Sukkur (Southern irrigated plain) and Thatta (Indus delta) in Sindh province (Figure 3.1; Table 3.1). Attempts were made to collect all ticks seen, however, it was not successful for some of the animals (particularly

buffaloes) due to restraining constraints under field conditions. Following collection, tick specimens from each animal were fixed and stored in 70% ethanol in separately labelled Eppendorf tubes. The collection of ticks was approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences of The University of Melbourne (ethics permit no. 714216).

3.3.2. Morphological characterisation

Each tick specimen was examined using a dissecting microscope (Olympus SZ40, Japan) and identified using dichotomous keys (Walker et al., 2003; Barker and Walker, 2014). Following morphological identification, all ticks representing the same species from the same host animal were pooled into individual Eppendorf tubes. In total, there were 234 tubes of pooled ticks from 204 animals for molecular characterisation.

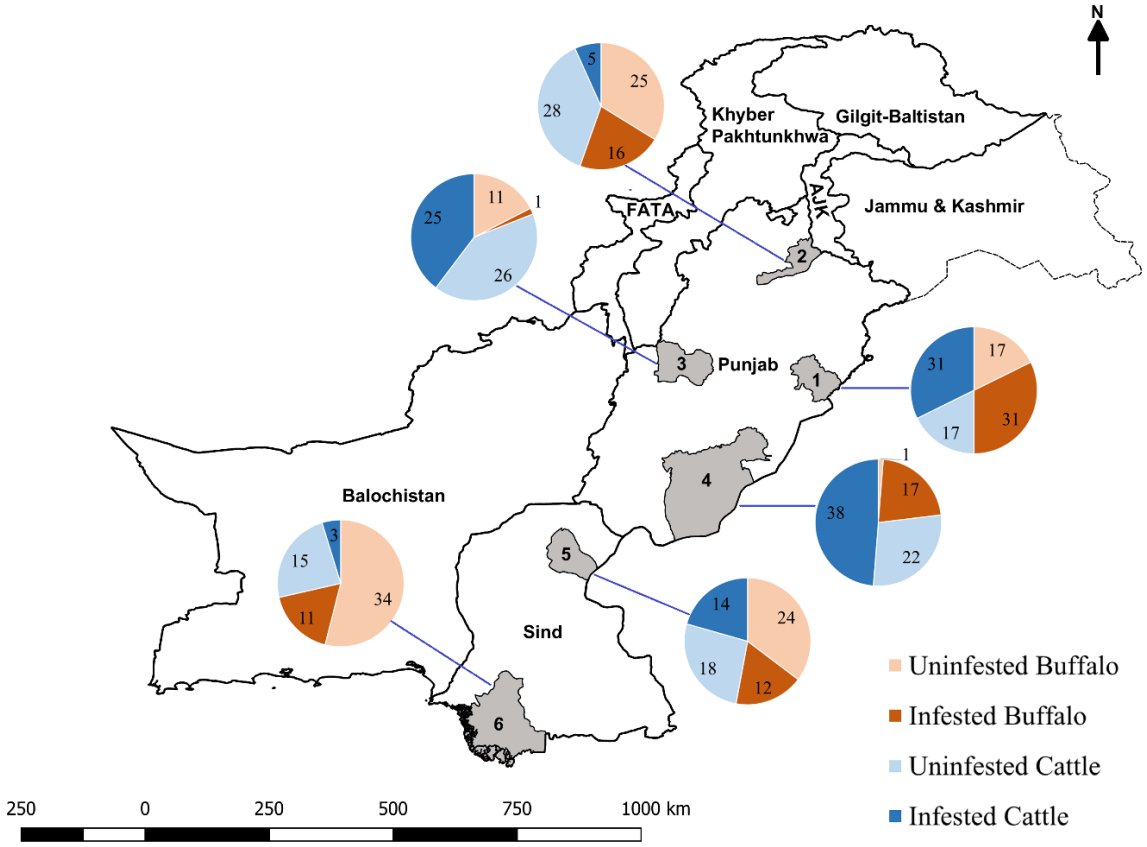


Figure 3.1. Map of Pakistan showing the number of tick-infested and un-infested cattle and buffaloes (Pie charts) from Okara (1), Jhelum (2), Layyah (3), Bahawalpur (4), Sukkur (5) and Thatta (6) districts.

Table 3.1. Details of locations and hosts for tick species detected in this study. GenBank accession numbers are also provided for the sequences of ticks determined herein

Districts (Coordinates)	**Host**	**Tick species**	**Number of tick life stages examined**			**GenBank accession numbers (only unique sequences were submitted)**		
			Adult		Nymph	16S	*cox*1	ITS-2
			Male	Female				
Okara (30.8138° N, 73.45334° E)	Buffalo	*Hyalomma* (*Hy*.) *anatolicum*	65	56	6	MN726552, MN726555	-	-
		Rhipicephalus (*Rh.*) *microplus*	0	2	0	-	-	-
	Cattle	*Hy. anatolicum*	49	67	7	-	MN728991, MN728995	-
		Rh. microplus	2	18	0	-	-	-
Jhelum (32.9425° N, 73.7257° E)	Buffalo	*Hy. anatolicum*	9	12	3	-	MN728996	-
		Hy. scupense	1	0	0	MN726557	MN729001	MN729007
		Rh. microplus	7	26	0	MN726558	MN729004, MN729003	MN729009
	Cattle	*Hy. anatolicum*	1	4	1	-	-	-
		Rh. microplus	0	11	0	-	-	-
		Rh. annulatus	1	0	0	MN726556	MN729002	MN729008
Bahawalpur (29.3544° N, 71.6911° E)	Buffalo	*Hy. anatolicum*	39	25	2	-	-	-
	Cattle	*Hy. anatolicum*	91	46	0	MN726551, MN726553	MN728992, MN728993	MN729005
Layyah (30.9693° N, 70.9428° E)	Buffalo	*Hy. anatolicum*	2	1	0	-	-	-
	Cattle	*Hy. anatolicum*	54	4	0	-	-	-
Sukkur (27.7244° N, 68.8228° E)	Buffalo	*Hy. anatolicum*	27	27	0	-	-	-
	Cattle	*Hy. anatolicum*	41	16	1	MN726554	MN728997	-
Thatta (24.7475° N, 67.9106° E)	Buffalo	*Hy. anatolicum*	21	13	1	-	MN728994	-
		Hy. hussaini	1	2	0	MN726588, MN726589	MN728999, MN729000	-
	Cattle	*Hy. anatolicum*	8	2	0	MN726550	-	-
		Hy. hussaini	2	0	0	MN726587	MN728998	MN729006
Total ticks			421	332	21			

3.3.3. Molecular characterisation

3.3.3.1. DNA extraction

Prior to DNA extraction, ethanol was removed, and one tick specimen from each pool (n = 234) was cut in half longitudinally. The one half of the tick was cut into four to six smaller pieces and washed three-times in milliQ water, and then cut finely with a scalpel blade. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, except that the proteinase K incubation at 56 °C was for 24-48 hours.

3.3.3.2. PCR amplification and DNA sequencing

Two mitochondrial (*cox*1 and 16S) and one nuclear (ITS-2) loci were separately amplified from each individual tick DNA sample employing previously published oligonucleotide primers (Black and Piesman, 1994; Vrijenhoek, 1994; Rees et al., 2003) using conventional PCR in a thermal cycler (T100, BioRad). Primer sequences and PCR conditions are given in Table 3.2. All PCRs were carried out in a final volume of 25 µl, containing 3.12 mM of each deoxynucleotide triphosphate (dNTP), 6.25 pmol of each primer, and 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 75 mM (16S), 100 mM (*cox*1) or 150 mM (ITS-2) MgCl2, and 0.6 U of GoTaq flexi DNA polymerase (Promega, Madison, WI, USA). Known positive (*Rh. sanguineus* sensu lato) and negative (milliQ H2O) controls were included in each PCR run. Aliquots (5 µl) of individual amplicons were examined on 1.5% (w/v) agarose gels stained with GelRed (Biotium) and photographed using a GelDoc system (BioRad, Hercules, CA, USA).

For each locus, amplicons were purified using Favorgen Gel/PCR purification mini kit (Favorgen, Taiwan), and the DNA concentration was measured using NanoDrop spectrophotometer (ND-1000, Wilmington, DE, USA). Automated, bidirectional (Sanger) sequencing of samples, employing the same primers (individually) used in PCR (Table 3.2), was conducted using an Applied Biosystems 3730 DNA Analyzer (Thermo Fisher Scientific, Waltham, MA USA).

Table 3.2. PCR primers and thermocycling conditions used for the amplification of three loci in this study

Target region	**Primers**	**PCR conditions**	**Amplicon size (base pairs)**	**Reference**
16S	Forward 16S+1 (5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3') Reverse 16S-1 (5'-CCGGTCTGAACTCAGATCAAGT-3')	Initial denaturation:95 °C, 4 min 35 cycles of Denaturation: 95 °C, 1 min Annealing: 50 °C, 1 min Extension: 68 °C, 1 min Final extension:68 °C, 10 min	460	(Black and Piesman, 1994)
*cox*1	Forward LCO1490 (5'- GGTCAACAAATCATAAAGATATTGG-3') Reverse HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3')	Initial denaturation: 95 °C, 5 min 40 cycles of Denaturation: 95 °C, 45 sec Annealing 50 °C, 45 sec Extension: 72 °C, 1 min Final extension: 72 °C, 5 min	710	(Vrijenhoek, 1994)
ITS-2	Forward RIB-8 (5'-GTCGTAGTCCGCCGTC-3') Reverse RIB-11 (5'-GAGTACGACGCCCTACC-3')	Initial denaturation: 94 °C, 5 min 35 cycles of Denaturation: 94 °C, 30 sec Annealing: 62 °C, 15 sec Extension: 72 °C, 15 sec Final extension: 72 °C, 5 min	280	(Rees et al., 2003)

3.3.4. Sequence and phylogenetic analyses

The quality of individual *cox*1, 16S and ITS-2 sequences was assessed. Forward and reverse sequences were inspected, and each consensus sequence assembled using Geneious Prime 2019.0.4 (www.geneious.com). The *cox*1 sequences were conceptually translated to ensure that the reading frame was open. All nucleotide sequences obtained were deposited in the GenBank (cf. Table 3.1).

Nucleotide sequences were aligned using MUSCLE v.3.8.31 (Edgar, 2004) within MEGA 7.0 using default settings (Kumar et al., 2016). Pairwise comparisons of aligned sequences were performed to calculate nucleotide differences using BioEdit (Hall, 1999). Sequences were then compared with published sequences in the National Center for Biotechnology Information (NCBI) using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to infer the specific identity of individual ticks. These published (reference) sequences represented *Hyalomma* and *Rhipicephalus* species recorded on bovines in Pakistan and other parts of South Asia.

Unique *cox*1, 16S and ITS-2 sequences representing all tick specimens collected in this study were aligned with respective reference sequences for each sequence. All alignments were performed using MUSCLE in MEGA using default settings and were trimmed to uniform lengths of 582 (*cox*1), 413 (16S) and 241 (ITS-2) bp. The evolutionary models for each DNA sequence dataset were determined using the Akaike and the Bayesian information criteria (AIC and BIC) tests in jModelTest v.3.7 (Darriba et al., 2012). Neighbour Joining (NJ) trees were constructed using MEGA, and Bayesian Inference (BI) trees were built using MrBayes plugin for Geneious (Huelsenbeck and Ronquist, 2001). The NJ trees were constructed with 10,000 bootstrap replicates for *cox*1 and 16S sequences using the Tamura-Nei distance method, and the p-distance method was used for aligned ITS-2 sequence data. Each BI analysis was run for 20,000,000 generations (ngen = 20,000,000) to calculate posterior probabilities (pp), with two runs, with every 200th tree saved (samplefreq = 200). In the analysis of *cox*1 sequence data, *Argas* (*A.*) *persicus* was employed as an outgroup; for 16S or ITS-2 data, *Haemaphysalis* (*Ha.*) *flava* was used.

Currently, at least five taxa of the *Rh. microplus* complex (A, B, C, *Rh. annulatus* and *Rh. australis*) have been defined based on unique *cox*1 sequences (Roy et al., 2018). Well-defined

*cox*1 reference sequences for each of these five taxa were used in the NJ and BI analyses to infer the identity of *Rhipicephalus* samples sequenced in this study.

3.3.5. Statistical analyses

The animal-level prevalence of ticks was calculated as the number of animals infested with ≥ 1 tick divided by the total number of animals examined. A herd was considered positive if ticks were found on at least one animal. The herd-level prevalence was calculated as the number of positive herds divided by total herds examined. The 95% CIs were calculated for binomial proportions using the package "binom" (Dorai-Raj, 2014) in R according to the Clopper-Pearson interval method (Clopper and Pearson, 1934). The overall tick prevalence (animal and herd levels) was compared between or among different AEZs and different age groups using the Chi-square test. The Fisher's exact test was employed to compare the prevalence of ticks between different host species (cattle and buffaloes) and genders (male and female). A *P*-value of < 0.05 was considered as statistically significant. Data were analysed using GraphPad 5 Prism program (GraphPad Software Inc., La Jolla, CA, USA).

3.4. Results

3.4.1. Morphological characterisation of ticks

The morphological identification revealed that 707 ticks (including nymphs) belonged to genus *Hyalomma* (681 *Hy. anatolicum*, 5 *Hy. hussaini*, 1 *Hy. scupense*, 20 unidentified species) and 67 ticks were from the genus *Rhipicephalus* (subgenus *Boophilus*) (66 *Rh. microplus* and 1 *Rh. annulatus*) (Table 3.1). Twenty *Hyalomma* specimens were *Hy. anatolicum* or *Hy. excavatum*, but morphological examination of these specimens did not allow a definitive species-level identification.

3.4.2. Prevalence of ticks

A total of 774 ticks (including 421 male, 332 female and 21 nymphs) was collected from 46.1% (204/442) of bovines (cattle: 116/242; buffaloes: 88/200) examined in this study (Table 3.1; Figure 3.1). The animal-level prevalence of ticks was significantly different among the districts ($P < 0.0001$), and the highest percentages of tick-infested bovines were in the district Bahawalpur (70.5%) and Okara (64.6%), followed by Layyah (41.3%), Sukkur (38.2%), Jhelum (28.4%) and Thatta (22.2%) (Figure 3.1). The herd-level prevalence of ticks was 59% (95% CI = 51-67) and varied significantly among the districts ($P < 0.0001$) (Figure 3.2). Highest percentages were in Okara (89%; 95% CI = 72-98) and Bahawalpur (84%; 95% CI = 64-95), followed by Thatta (54%; 95% CI = 33-73), Layyah (44%; 95% CI = 24-65), Jhelum (36%; 95% CI = 18-57) and Sukkur (35%; 95% CI = 17-56) (Figure 3.2). The overall prevalence of ticks in male and female bovines was 45% and 46.5%, respectively, and was slightly higher in cattle (47.9%) than in buffaloes (44%), but this difference was statistically non-significant ($P > 0.05$). Tick prevalence was highest in calves (< 1 year of age) (55%, 38/69; 95% CI = 43-67), followed by adults (> 3 years of age) (48%, 113/237; 95% CI = 41-54) and young animals (1-3 years of age) (39%, 53/136; 95% CI = 31-48) (Figure 3.2).

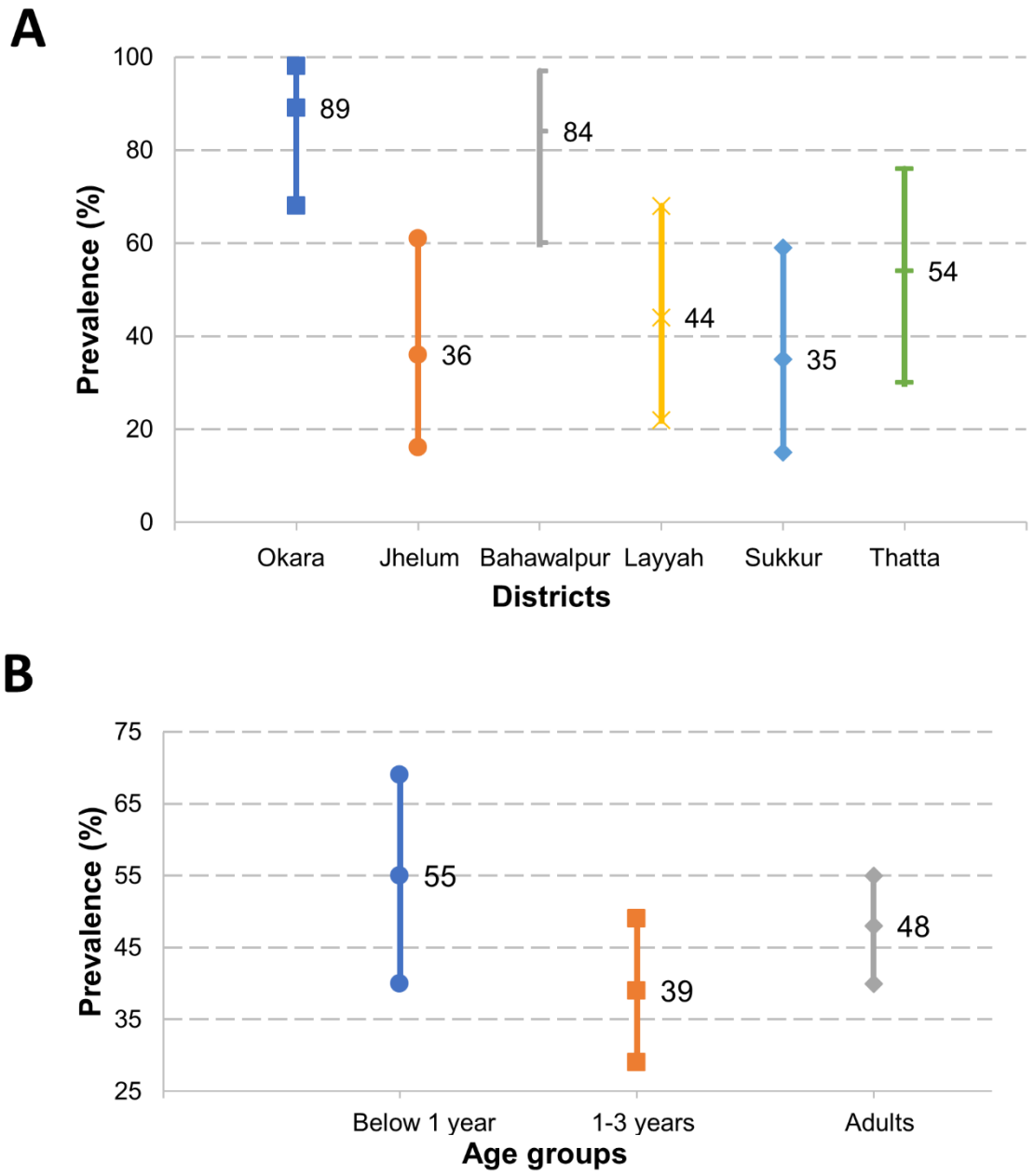


Figure 3.2. Overall prevalence of bovine ticks at a herd-level (A) and in different age groups of cattle and buffaloes (B) from six districts of Pakistan. Each middle point show percentage prevalence of ticks while the vertical line shows upper and lower 95% confidence intervals.

3.4.3. Sequence and phylogenetic analyses

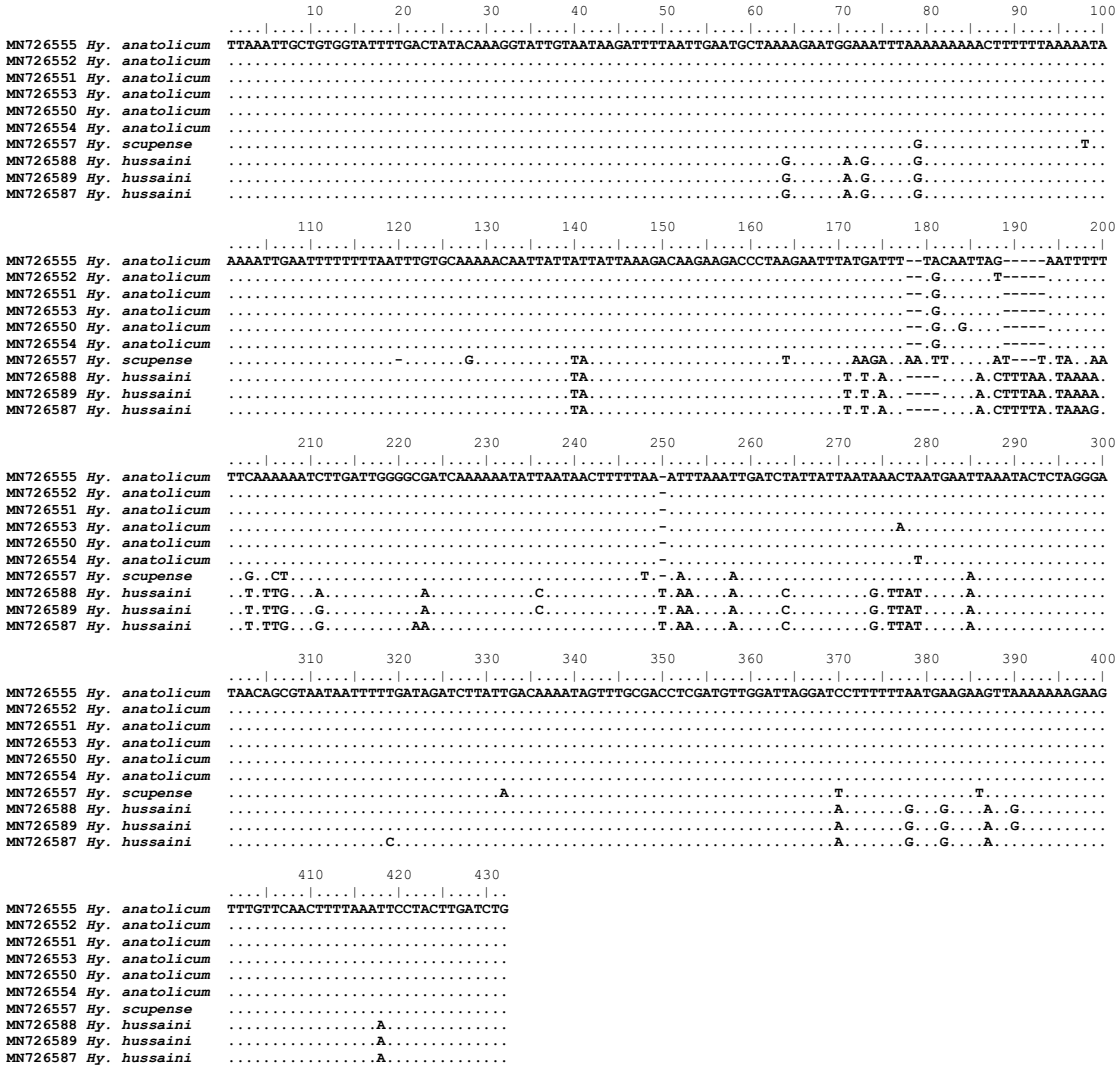
PCR amplification of each of the three loci was achieved from DNA isolated from 234 individual ticks. For DNA sequencing and phylogenetic analyses, 52 amplicons representing each of the three loci and all tick species from each host species and district as well as those with an indeterminant species status were selected for sequencing. The sequences obtained represented *Hyalomma* (n = 39) and *Rhipicephalus* (n = 13); and confirmed the morphological identification (data not shown). All morphologically indistinguishable tick specimens were molecularly identified as *Hy. anatolicum*.

The 16S, *cox*1 and ITS-2 sequences derived from *Hyalomma* were 432 bp, 650 bp and 268 bp, respectively (Figure 3.3 (A-C)), and those from *Rhipicephalus* were 432 bp, 582 bp and 260 bp, respectively (Figure 3.3 (D-F)). Upon pairwise comparison of homologous DNA sequences for *Hyalomma* specimens, the highest percentage differences were recorded in ITS-2 (9.1-16.5%), followed by *cox*1 (0.2-14.2%) and 16S (0.3-11.6%) (Tables 3.3-3.5). For *Rhipicephalus* specimens, the highest sequence differences were in *cox*1 (0.4-6.1%), followed by ITS-2 (2%) and 16S (1.7%) (Tables 3.6-3.8). Within the genus *Hyalomma*, sequences from *Hy. hussaini* had higher levels of difference among individual specimens (16S: 0.3-1.7%; *cox*1: 0.4-2.4%) than among those of *Hy. anatolicum* (*cox*1: 0.2-0.5%; 16S: 0.3-0.5%). For *Rh. microplus*, a nucleotide difference of 0.4% was observed exclusively in 16S, with no variation detected for other loci (Tables 3.3-3.8).

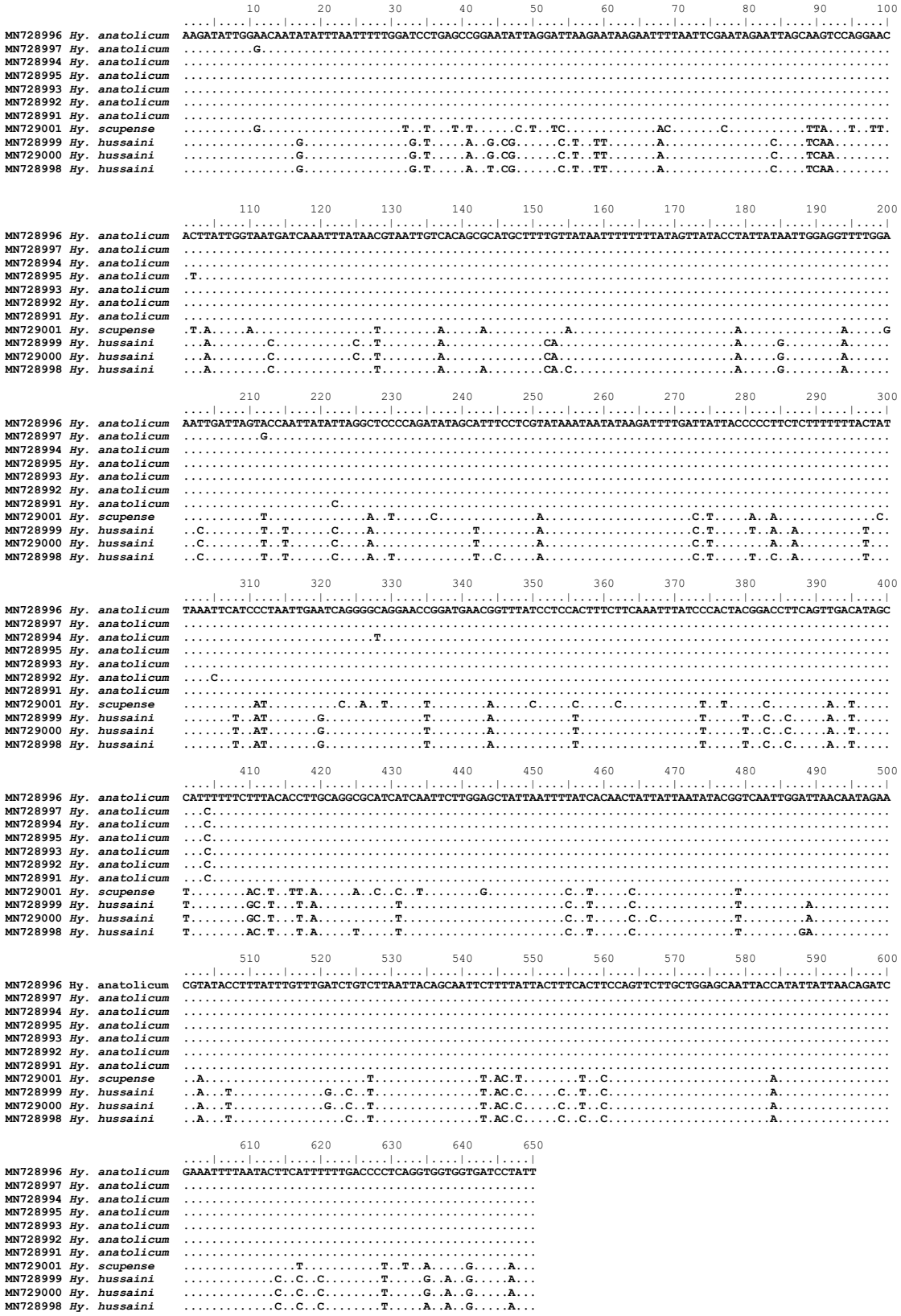
Molecular phylogenetic trees constructed separately using 16S, *cox*1 or ITS-2 sequence data employing the NJ and BI methods had similar topologies. Therefore, representative NJ trees, along with nodal support values for both methods are presented here for all three loci (Figures 3.4-3.6). Phylogenetic analyses revealed two distinct clades: one for *Hyalomma* spp. and the other for *Rhipicephalus* spp. (Figures 3.4-3.6). Within the first clade (Figures 3.4-3.6), all *Hy. anatolicum* sequences determined here (GenBank accession nos. 16S: MN726550-MN726555; *cox*1: MN728991-MN728997; ITS-2: MN729005) grouped with those previously published from Pakistan, with moderate to strong statistical support (16S: posterior probabilities for BI = 0.96, bootstrap for NJ = 78%; *cox*1: 0.81, 98%; ITS-2: 0.96, 99%). Sequences of a tick specimen from Jhelum district (16S: MN726557; *cox*1: MN729001; ITS-2: MN729007) grouped with *Hy. scupense* from Pakistan with moderate to strong statistical support (16S: 1, 100%; *cox*1: 1, 100%; ITS-2: 0.77, 100%). Sequences from

ticks morphologically identified as *Hy. hussaini* from Thatta district (16S: MN726587-MN726589; *cox*1: MN728998-MN729000; ITS-2: MN729006) grouped with *Hy. hussaini* from Pakistan (Sands et al., 2017) with strong statistical support (16S: 0.91, 99%; ITS-2: 0.95, 99%). No *cox*1 sequence for *Hy. hussaini* was available in public databases; hence, the three *cox*1 sequences obtained herein each grouped closer to *Hy. kumari* from Pakistan (KU130607), (Sands et al., 2017), (Figure 3.5). Within the *Rhipicephalus* clade, one sequence from Jhelum district (16S: MN726556; *cox*1: MN729002; ITS-2: MN729008) grouped with sequences of *Rh. annulatus* previously reported from the USA (16S), India (*cox*1), and Israel (ITS-2), with moderate to strong nodal support (16S: 0.85, 98%; *cox*1: 1, 100%; ITS-2: 1, 88%). All other sequences of *Rhipicephalus* ticks determined here (16S: MN726558; *cox*1: MN729003, MN729004; ITS-2: MN729009) grouped with *Rh. microplus* reported from India (16S), Pakistan (*cox*1) and China (ITS-2) with varying levels of support (16S: 0.61, 83%; *cox*1: 1, 100%; ITS-2: 0.93, 74%). For the ITS-2 trees, the topology was similar to those obtained using data for each of the two mitochondrial loci (16S and *cox*1; Figures 3.4-3.5), except for one sequence for *Hy. anatolicum* (MN729005) which grouped with those previously published from Pakistan (KU130744), (Sands et al., 2017) as well as *Hy. excavatum* from Israel (KU130755), (Sands et al., 2017), with strong nodal support (0.96, 99%) (Figure 3.6).

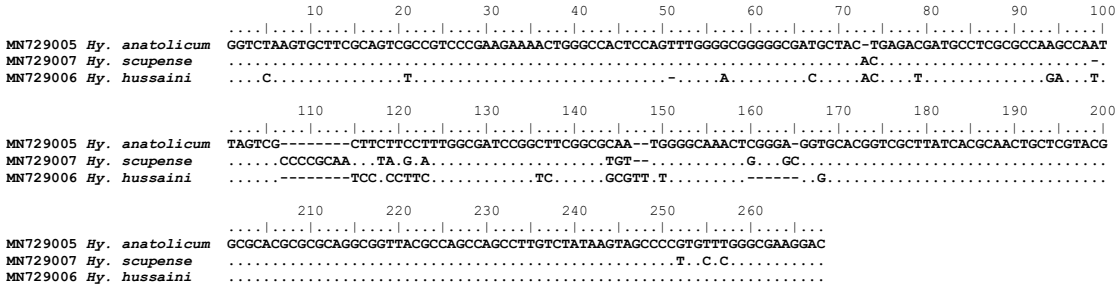
A: 16S nucleotide alignment: Genus *Hyalomma*



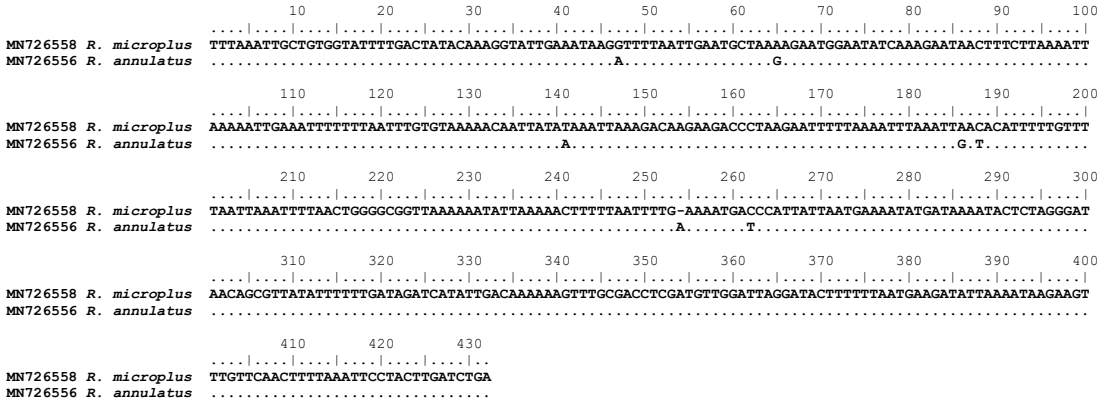
B: *cox*1 nucleotide alignment: Genus *Hyalomma*



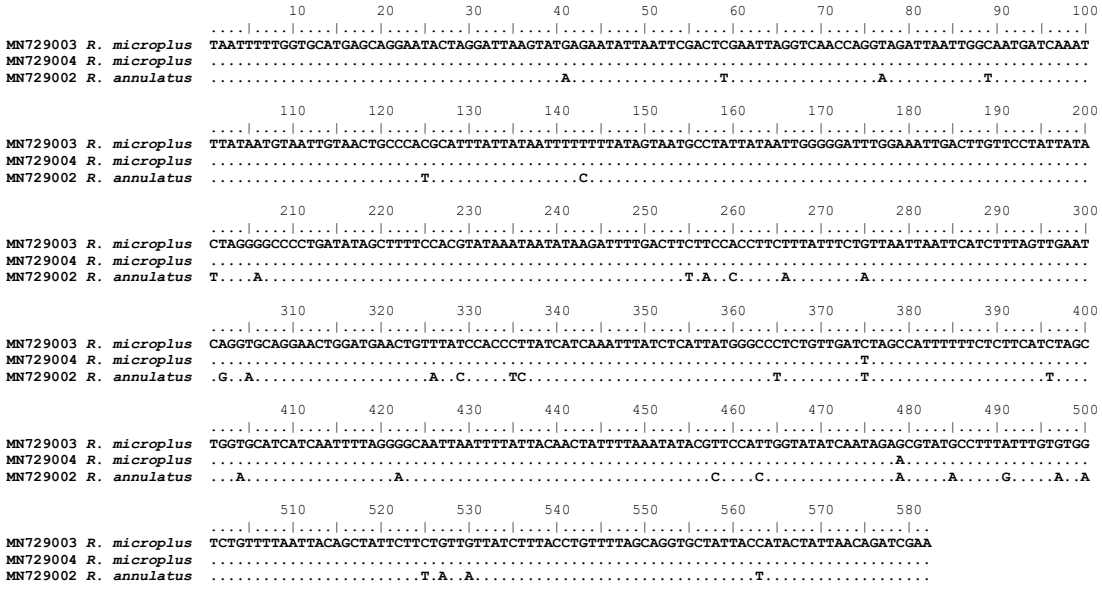
C: ITS-2 nucleotide alignment: Genus *Hyalomma*



D: 16S nucleotide alignment: Genus *Rhipicephalus*



E: *cox*1 nucleotide alignment: Genus *Rhipicephalus*



F: ITS-2 nucleotide alignment: Genus *Rhipicephalus*

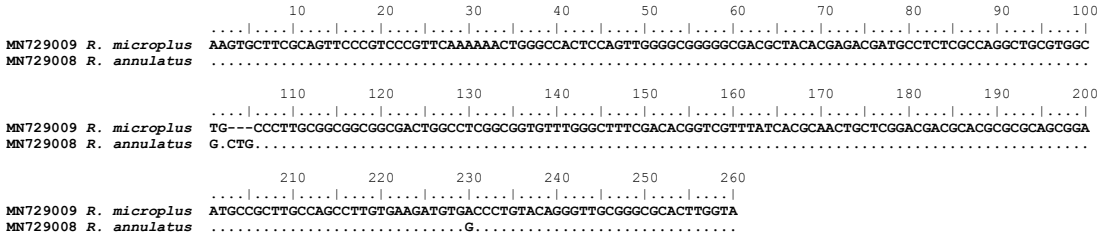


Figure 3.3 (A-F). Separate nucleotide alignments of the 16S, *cox*1 and ITS-2 sequences of *Hyalomma* (A-C) and *Rhipicephalus* (D-F) specimens, respectively. A dot indicates an identical nucleotide with respect to the top sequence.

Table 3.3. Pairwise comparison of 16S nucleotide sequences (aligned over 432 bp) of tick specimens belonging to the genus *Hyalomma* determined herein. Nucleotide similarity and percentage differences are given above and below the diagonal, respectively.

	GenBank ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	MN726555 (*Hy. anatolicum*)		0.995	0.997	0.995	0.995	0.995	0.925	0.89	0.89	0.89
2	MN726552 (*Hy. anatolicum*)	0.5		0.997	0.995	0.995	0.995	0.925	0.89	0.89	0.89
3	MN726551 (*Hy. anatolicum*)	0.3	0.3		0.997	0.997	0.997	0.925	0.89	0.89	0.89
4	MN726553 (*Hy. anatolicum*)	0.5	0.5	0.3		0.995	0.995	0.922	0.89	0.89	0.89
5	MN726550 (*Hy. anatolicum*)	0.5	0.5	0.3	0.5		0.995	0.922	0.888	0.888	0.888
6	MN726554 (*Hy. anatolicum*)	0.5	0.5	0.3	0.5	0.5		0.922	0.893	0.893	0.893
7	MN726557 (*Hy. scupense*)	7.5	7.5	7.5	7.8	7.8	7.8		0.886	0.886	0.884
8	MN726588 (*Hy. hussaini*)	11	11	11	11	11.2	10.7	11.4		0.997	0.983
9	MN726589 (*Hy. hussaini*)	11	11	11	11	11.2	10.7	11.4	0.3		0.985
10	MN726587 (*Hy. hussaini*)	11	11	11	11	11.2	10.7	11.6	1.7	1.5	

Table 3.4. Pairwise comparison of *cox*1 nucleotide sequences (aligned over 650 bp) of tick specimens belonging to the genus *Hyalomma* determined herein. Nucleotide similarity and percentage differences are given above and below the diagonal, respectively.

	GenBank ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	MN728995 (*Hy. anatolicum*)		0.996	0.996	0.998	0.996	0.996	0.995	0.87	0.864	0.864	0.858
2	MN728991 (*Hy. anatolicum*)	0.4		0.996	0.998	0.996	0.996	0.995	0.867	0.867	0.867	0.861
3	MN728996 (*Hy. anatolicum*)	0.4	0.4		0.998	0.996	0.996	0.995	0.87	0.867	0.867	0.861
4	MN728993 (*Hy. anatolicum*)	0.2	0.2	0.2		0.998	0.998	0.996	0.869	0.866	0.866	0.86
5	MN728992 (*Hy. anatolicum*)	0.4	0.4	0.4	0.2		0.996	0.995	0.867	0.864	0.864	0.858
6	MN728994 (*Hy. anatolicum*)	0.4	0.4	0.4	0.2	0.4		0.995	0.867	0.864	0.864	0.858
7	MN728997 (*Hy. anatolicum*)	0.5	0.5	0.5	0.4	0.5	0.5		0.87	0.864	0.864	0.858
8	MN729001 (*Hy. scupense*)	13	13.3	13	13.1	13.3	13.3	13		0.887	0.886	0.89
9	MN728999 (*Hy. hussaini*)	13.6	13.3	13.3	13.4	13.6	13.6	13.6	11.3		0.996	0.98
10	MN729000 (*Hy. hussaini*)	13.6	13.3	13.3	13.4	13.6	13.6	13.6	11.4	0.4		0.976
11	MN728998 (*Hy. hussaini*)	14.2	13.9	13.9	14	14.2	14.2	14.2	11	2	2.4	

Table 3.5. Pairwise comparison of ITS-2 nucleotide sequences (aligned over 268 bp) of tick specimens belonging to the genus *Hyalomma* determined herein. Nucleotide similarity and percentage differences are given above and below the diagonal, respectively.

	GenBank ID	**1**	**2**	**3**
1	MN729005 (*Hy. anatolicum*)		0.909	0.872
2	MN729007 (*Hy. scupense*)	9.1		0.835
3	MN729006 (*Hy. hussaini*)	12.8	16.5	

Table 3.6. Pairwise comparison of 16S nucleotide sequences (aligned over 432 bp) of tick specimens belonging to the genus *Rhipicephalus* determined herein. Nucleotide similarity and percentage differences are given above and below the diagonal, respectively.

	GenBank ID	**1**	**2**
1	MN726558 (*Rh. microplus*)		0.983
2	MN726556 (*Rh. annulatus*)	1.7	

Table 3.7. Pairwise comparison of *cox*1 nucleotide sequences (aligned over 582 bp) of tick specimens belonging to the genus *Rhipicephalus* determined herein. Nucleotide similarity and percentage differences are given above and below the diagonal, respectively.

	GenBank ID	**1**	**2**	**3**
1	MN729003 (*Rh. microplus*)		0.996	0.939
2	MN729004 (*Rh. microplus*)	0.4		0.943
3	MN729002 (*Rh. annulatus*)	6.1	5.7	

Table 3.8. Pairwise comparison of ITS-2 nucleotide sequences (aligned over 260 bp) of tick specimens belonging to the genus *Rhipicephalus* determined herein. Nucleotide similarity and percentage differences are given above and below the diagonal, respectively.

	GenBank ID	**1**	**2**
1	MN729009 (*Rh. microplus*)		0.98
2	MN729008 (*Rh. annulatus*)	2	

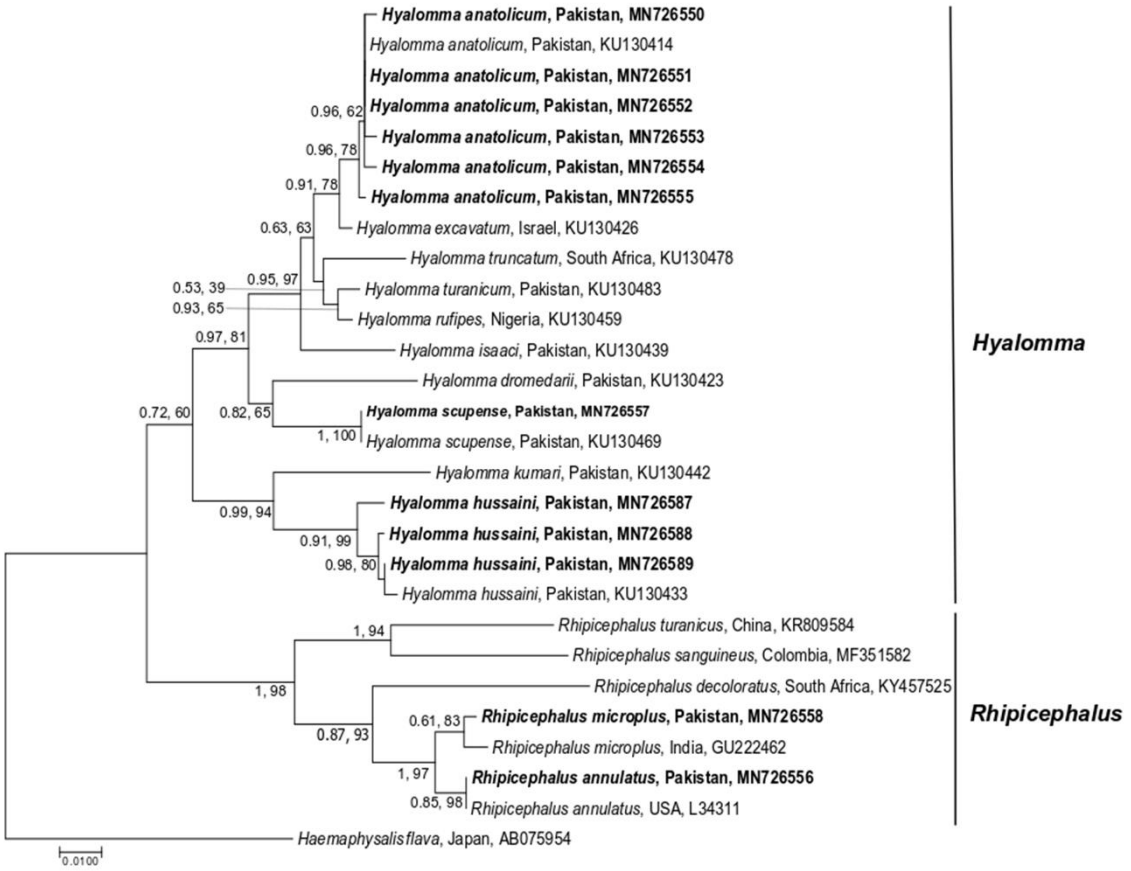


Figure 3.4. Genetic relationships of *Hyalomma* spp. and *Rhipicephalus* spp. specimens from Pakistan (bold) with reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analyses of 16S gene partial sequence data using Neighbour Joining (NJ, this tree) and Bayesian Inference (BI, not shown) methods, and *Haemaphysalis flava* was used as the outgroup. The country of origin and GenBank accession numbers for each sequence are also provided. Nodal support values are indicated: posterior probability for BI (left) and bootstrap value for NJ (right). The scale bar indicates distance.

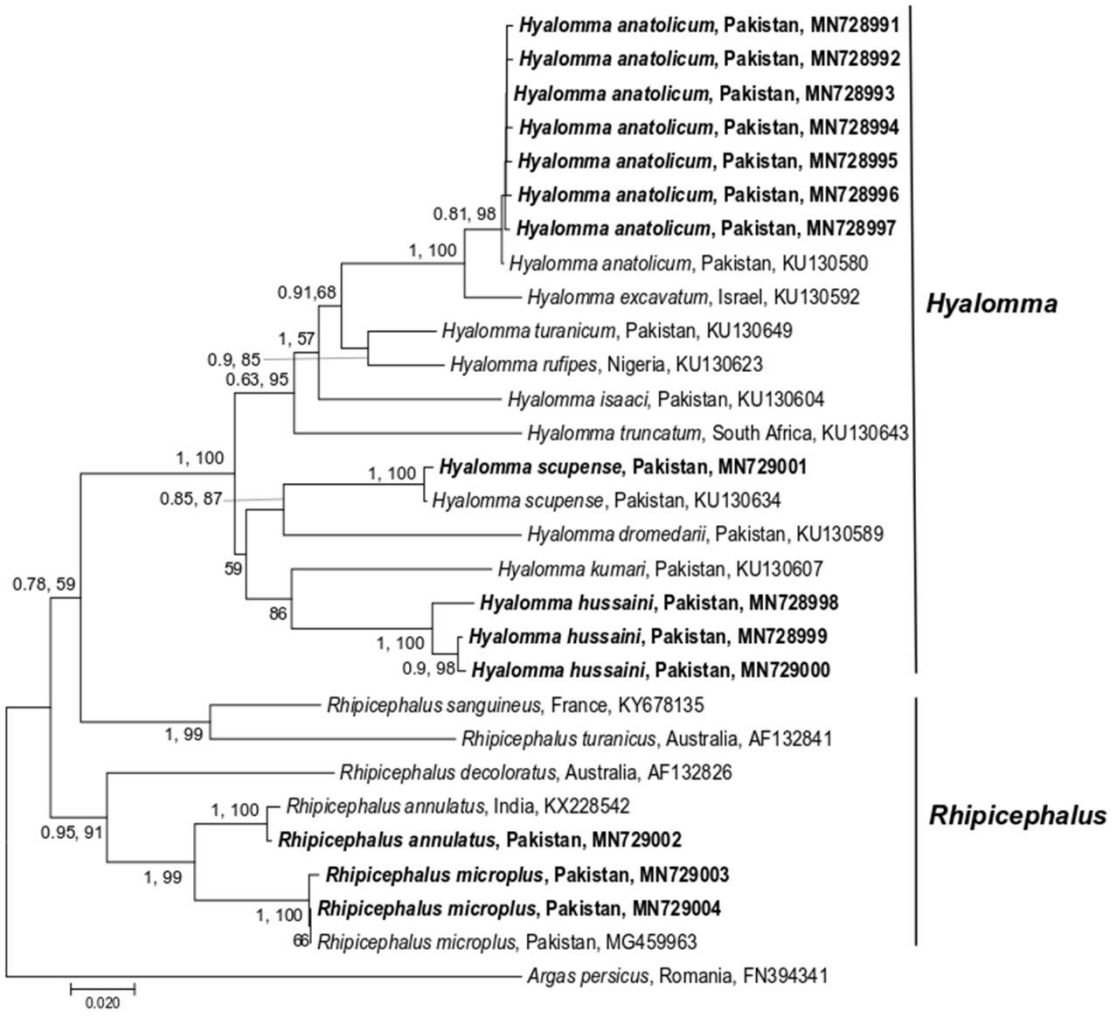


Figure 3.5. Genetic relationships of *Hyalomma* spp. and *Rhipicephalus* spp. isolates from Pakistan (bold) with reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analyses of cox1 gene partial sequence data using Neighbour Joining (NJ, this tree) and Bayesian Inference (BI, not shown) methods, and *Argas persicus* was used as the outgroup. The country of origin and GenBank accession numbers for each sequence are also provided. Nodal support values are indicated: posterior probability for BI (left) and bootstrap value for NJ (right). The scale bar indicates distance.

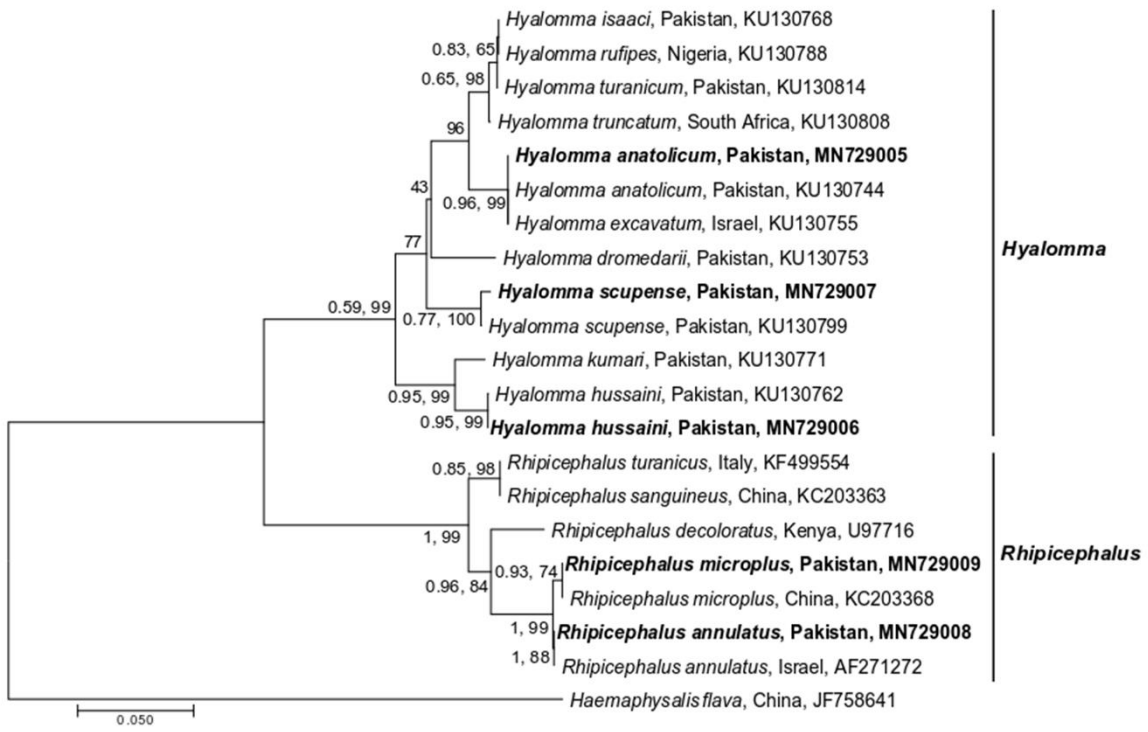


Figure 3.6. Genetic relationships of *Hyalomma* spp. and *Rhipicephalus* spp. specimens from Pakistan (bold) with reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analyses of ITS-2 region partial sequence data using Neighbour Joining (NJ, this tree) and Bayesian Inference (BI, not shown) methods, and *Haemaphysalis flava* was used as the outgroup. The country of origin and GenBank accession numbers for each sequence are also provided. Nodal support values are indicated: posterior probability for BI (left) and bootstrap value for NJ (right). The scale bar indicates distance.

To establish which of the five clades of ticks that our sequences of *Rhipicephalus* spp. represented (Roy et al., 2018), only *cox*1 sequences were subjected to analysis using BI and NJ methods. The topology of the BI and NJ trees was similar; hence, only the NJ tree is presented here (Figure 3.7). Two *cox*1 sequences representing *Rh. microplus* (MN729003, MN729004) grouped with those published previously from Pakistan ((MG459963), (Roy et al., 2018)) and India (KP792578, KP792579 and KP792587, unpublished) with absolute nodal support (1, 100%), whereas the third *cox*1 sequence of *Rhipicephalus* (MN729002) clustered with four sequences from *Rh. annulatus* from Iraq (KM235716, unpublished), Iran ((KM494917), (Ronaghi et al., 2015)) and Israel ((AF132825), (Murrell et al., 2000) and KF219739, unpublished)) with variable nodal support (0.63, 99%) (Figure 3.7). Clade B represented *Rh. microplus* from China, and clade A represented *Rh. microplus* from Brazil, Cambodia and Panama, whereas the *Rh. australis* clade represented ticks from Australia (Figure 3.7).

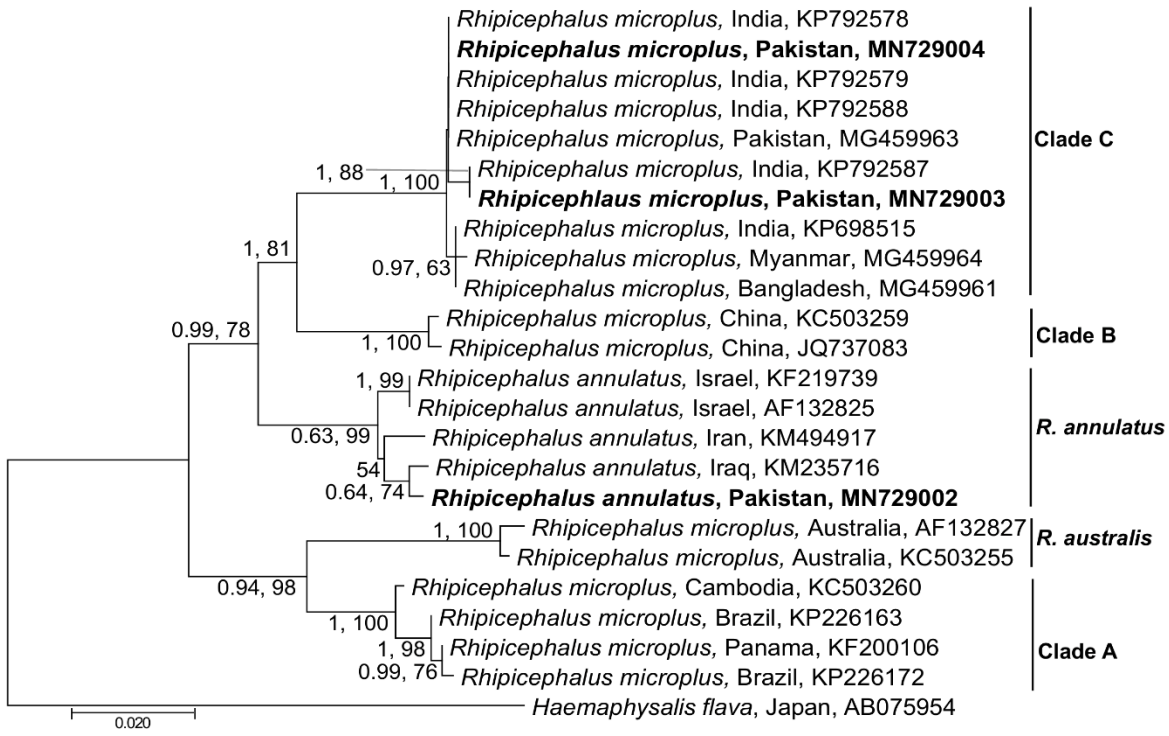


Figure 3.7. Genetic relationships of *Rhipicephalus microplus* complex spp. isolates from Pakistan (bold) with reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analyses of *cox*1 gene partial sequence data using Neighbour Joining (NJ, this tree) and Bayesian Inference (BI, not shown) methods, and *Haemaphysalis flava* was used as the outgroup. The country of origin and GenBank accession numbers for each sequence are also provided. Nodal support values are indicated: posterior probability for BI (left) and bootstrap value for NJ (right). The scale bar indicates distance.

3.5. Discussion

This is the first morphological-molecular phylogenetic study of bovine ixodid ticks from five AEZs in Pakistan. It is also the first record of *Rh. annulatus* in Pakistan. Previous investigations in Pakistan relied on the morphological identification of ticks (Sajid et al., 2008; Irshad et al., 2010; Iqbal et al., 2013; Farooqi et al., 2017; Karim et al., 2017), with four studies employing genetic tools to characterise small numbers of ticks from a limited number of geographical locations (Rehman et al., 2017; Roy et al., 2018; Ali et al., 2019; Zeb et al., 2019).

The prevalence of ticks significantly varied among different AEZs (range = 22.2-70.5%; $P < 0.0001$), and the highest tick prevalence was found in Bahawalpur (Sandy desert) and Okara (Northern irrigated plain) districts - both of which have the second highest cattle and buffalo populations, respectively, in Pakistan (Livestock & Dairy Development Department, 2018). Bahawalpur district is located in the hottest climatic zone with an annual rainfall of ≥ 23 mm, while Okara is in a relatively mild climatic zone, with an annual rainfall of ≥ 67 mm (Salma et al., 2012). Previous studies from Pakistan also reported variation in tick infestation among different regions of the country (Sajid et al., 2009; Ahmad et al., 2019). However, these studies did not include tick specimens from bovines located in different AEZs of Pakistan. To the best of our knowledge, there is only one study by Rehman et al. (2017) who addressed AEZs based on aridity index and found significantly lower tick prevalence in the semi-arid zone (72%) than arid zone (81%). The variation in the prevalence of ticks could relate to differences (i) in climatic conditions among different AEZs and (ii) management and husbandry practices used by small-holder dairy farmers across the study districts, as the distribution of host population and abiotic biogeography play an important role in the evolution and distribution of ticks (Klompen et al., 1996; Mccoy et al., 2013; Estrada-Pena and de la Fuente, 2014; Dantas-Torres, 2015; Ogden and Lindsay, 2016).

In this study, the prevalence of ticks differed even within the same AEZ (i.e. arid: districts Jhelum vs Layyah), which could relate to topographic differences between both districts. The Jhelum district is in the Pothohar Plateau along the river Jhelum and has a sub-mountainous topography, whereas the Layyah district is located in the southern part of Punjab province representing the plain lands and the Thal desert. The topography of a region can have an impact on husbandry practices (linked to land use), the distribution and types of livestock

species as well as climatic conditions, which can associate with tick prevalence in an area. Previously, a study reported the prevalence of ticks from two districts (i.e. Layyah and Muzaffargarh) in Punjab, Pakistan and recorded the variation of tick prevalence between the districts (Sajid et al., 2009). However, in contrast to the tick prevalence of 41.2% for Layyah district (investigated during November 2017), Sajid et al. (2009) did not find any ticks on bovines in this district from November to March. This difference could be due to a variation in (i) study design, as we targeted small-holder dairy farms, whereas Sajid et al. (2009) included only herds with ≥ 10 animals – farms with better management practices and regular use of acaricidal drugs (Ghosh et al., 2007), and (ii) variation in climatic conditions (i.e. annual temperature and rainfall) (Dantas-Torres, 2015; Ogden and Lindsay, 2016).

We observed a higher prevalence of ticks in calves than both juveniles and adults which is in agreement with a study from the Indian Punjab (Singh and Rath, 2013), but in contrast to the findings of Rehman et al. (2017) and Sajid et al. (2009) from Pakistan. There are also other contrasting reports in the literature about the susceptibility of different age groups of bovines to the prevalence and intensity of ticks (Sherrard-Smith et al., 2012; Anderson et al., 2013). The lower prevalence of ticks in adult animals could relate to the acquired immunity following repeated exposure to ticks (Lew-Tabor et al., 2017) and better care of these animals than calves - which are more susceptible to infections due to limited immunity against ticks and usually receive less attention in small-scale farm establishments (Singh and Rath, 2013; Burrow et al., 2019).

In this study, *Hy. anatolicum* was the most abundant tick species (90.6%; 701/774) of cattle and buffaloes in all AEZs, which is in accordance with findings of some previous studies from Pakistan (Sajid et al., 2009; Karim et al., 2017; Rehman et al., 2017) and India (Chhillar et al., 2014). *Hyalomma anatolicum* mainly infests bovine species and follows a three-host life cycle (Vatansever, 2017), and is widely distributed in Afrotropical, Oriental and Palearctic regions (Guglielmone et al., 2014). It is morphologically indistinguishable from *Hy. excavatum* - a closely related species, with an as yet indeterminate classification (Delpy, 1949; Hoogstraal and Kaiser, 1959; Guglielmone et al., 2014), as also demonstrated in this study. *Hyalomma anatolicum* is also known to transmit Crimean-Congo haemorrhagic fever (CCHF) from humans or livestock animals, including cattle, sheep and goats, to humans in various parts of Pakistan (Atif et al., 2017; Rehman et al., 2018; Yaqub et al., 2019) and

other parts of the world (Bakheit et al., 2012; Mourya et al., 2012; Al-Abri et al., 2017; Manjunathachar et al., 2019).

The second most abundant species found herein was *Rh. microplus* (8.5%; 66/774) in the Okara and Jhelum districts. These findings are consistent with Rehman et al. (2017) who also reported an absence of this tick species in drier districts of Punjab, Pakistan. Similarly, Singh and Rath (2013) reported that *Rh. microplus* prefers habitats with a hot and humid climate in India. *Rhipicephalus microplus*, a one-host tick is economically the most important tick species of bovines globally and is a known vector for economically important TBDs of bovines (i.e. anaplasmosis and babesiosis) (Lew-Tabor et al., 2017).

In this study, *Hy. hussaini* was found in low numbers (0.65%; 5/774) in cattle in Thatta district. It is a three-host tick with a distribution limited to South Asia, but little is known about its bionomics (Kaiser and Hoogstraal, 1964) and its vector competence also remains unexplored/unknown to date. Kaiser and Hoogstraal (1964) identified this species, for the first time, in the Indian subcontinent, and recently, Karim et al. (2017) recorded it from camels in Balochistan province of Pakistan. Here, we provide the first *cox*1 partial sequences for *Hy. hussaini*. We also found *Hy. scupense* (0.13%; 1/774) and *Rh. annulatus* (0.13%; 1/774) in Jhelum district of Punjab. Both of these species have been reported previously from other parts of Pakistan (Karim et al., 2017). However, we provide the first genetic characterisation of *Rh. annulatus* from Pakistan as there was no genetic evidence of the occurrence of this species in Pakistan or neighbouring countries (Roy et al., 2018). *Rhipicephalus annulatus* belongs to the *Rh. microplus* complex and is capable of transmitting anaplasmosis and babesiosis (Lew-Tabor et al., 2017). *Hyalomma scupense* is a one or two-host tick capable of infesting several host species and transmitting important pathogens such as *T. annulata*, *T. equi*, *Coxiella* (*C.*) *burnetii* and CCHF virus (Gharbi and Darghouth, 2014). Due to the recent reports of Q fever from Pakistan (Shabbir et al., 2016), future studies are required to establish a role of *Hy. scupense* in the transmission of *C. burnetii* in Pakistan.

3.6. Conclusions

This study provides a morphological and genetic characterisation of five species of bovine ixodid ticks from five AEZs of Pakistan. Our findings reveal that *Hy. anatolicum* and *Rh. microplus* are the two most abundant ticks of cattle and buffaloes in Pakistan and that their distribution varies considerably across different AEZs of the country. This study provides the first genetic evidence for the occurrence of *Rh. annulatus* in Pakistan. Further investigations are required for the in-depth monitoring and prevention of TTBDs involving a larger population of bovines across different seasons in Pakistan.

3.7. References

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CHAPTER 4. BOVINE TICKS HARBOUR A DIVERSE ARRAY OF MICROORGANISMS IN PAKISTAN

4.1. Abstract

Ticks and tick-borne pathogens (TTBPs) are a major constraint to livestock production in Pakistan; despite a high prevalence of TTBPs, knowledge on the capacity of Pakistani ticks to carry pathogens and endosymbionts is limited. Furthermore, mixed infections with multiple microorganisms further complicate and limit the detection potential of traditional diagnostic methods. The present study investigated the tick-borne microorganisms in bovine ticks in Pakistan, employing a high-throughput microfluidic real-time PCR based technique. Ticks were collected from clinically healthy cattle (n = 116) and water buffaloes (n = 88) from 30 villages across six districts located in five agro-ecological zones (AEZs) of Pakistan from September to November 2017. The microfluidic real-time PCR was used to test the genomic DNA of individual ticks for the presence of 27 bacterial and eight parasitic microorganisms. Phylogenetic methods were used to assess the genetic relationship of DNA sequences determined herein. PCR detected DNA of at least one microorganism in each of 221 ticks tested (94.4%, 221/234). DNA-based detection inferred that single pathogens/endosymbionts were the most common (43.4%, 96/221) followed by double (38.9%, 86/221), triple (14.5%, 32/221), quadruple (2.3%, 5/221) and quintuple (0.9%, 2/221) mixed infections. Piroplasms (*Babesia*/*Theileria* spp.) were the most prevalent (31.6%, 74/234), followed by *Ehrlichia* spp. (20%, 47/234) and *Anaplasma marginale* (7.7%, 18/234). *Anaplasma phagocytophilum*, *A. ovis*, *A. centrale*, *Babesia ovis*, *Borrelia* spp., *Rickettsia* spp., *R. massiliae*, *Bartonella* spp. and *Hepatozoon* spp. were also detected. Endosymbionts such as *Francisella*-like (91.5%, 214/234) and *Coxiella*-like (1.3%, 3/234) organisms were also detected in ticks. The highest diversity of microorganisms was detected in *Hyalomma anatolicum* ticks (test-positive for 14/14 microorganisms), followed by *Rhipicephalus microplus* (4/14), *Hy. hussaini* (3/14) and *Rh. annulatus* (2/14). Ticks collected from cattle carried significantly more frequently piroplasms (41.2%, 54/131; P < 0.05) than those from buffaloes (19.4%, 20/103). However, the overall prevalence of

microorganisms did not vary significantly among ticks from the two host species as well as across different AEZs. To our knowledge, this is the first study to investigate a wide range of tick-borne microorganisms in bovine ticks using a high-throughput diagnostic method from different AEZs in Pakistan. These findings will aid in establishing the distribution patterns and the control of tick-borne pathogens of bovines in Pakistan.

4.2. Introduction

The occurrence and prevalence of tick-borne pathogens (TBPs) in bovines have been reported from different parts of Pakistan (Irshad et al., 2010; Qayyum et al., 2010; Atif et al., 2012; Bhutto et al., 2012; Naz et al., 2012; Ali et al., 2013; Ashraf et al., 2013; Atif et al., 2013; Iqbal et al., 2013; Khan et al., 2013; Ahmad et al., 2014; Sajid et al., 2014; Farooqi et al., 2017; Karim et al., 2017; Hassan et al., 2018a; Hassan et al., 2018b; Rehman et al., 2019). However, most of these studies have utilised conventional diagnostic methods for the detection of TBPs in bovines. Although economical and useful for diagnosing clinical cases, these methods have lower sensitivity and specificity (Salih et al., 2015). Furthermore, previous studies were confined to the surrounding areas of major metropolitan cities without considering diverse climatic conditions and bovine production systems in Pakistan which are important factors in the design of epidemiological studies (Jabbar et al., 2015). Recently, several studies have utilised molecular methods for the detection of pathogens in bovines (Hassan et al., 2018a; Hassan et al., 2018b) and their ticks (Rehman et al., 2019). However, these studies only targeted the main TBPs pathogens (e.g. *Anaplasma* spp., *Babesia* spp. and *Theileria* spp.). As ticks usually can carry and transmit multiple pathogens, several non-pathogenic commensals and mutualistic microorganisms, known as endosymbionts, concurrently (Duron et al., 2015), it is important to explore these non-pathogenic organisms as well because they may interact with pathogens and evolve over time to become pathogenic to humans and/or animals (e.g. *Coxiella burnetii*) (Michelet et al., 2014; Duron et al., 2015; Moutailler et al., 2016).

In the last decade, various TBPs using molecular methods have been reported from bovines in Pakistan (Karim et al., 2017; Hassan et al., 2018a; Hassan et al., 2018b; Rehman et al., 2019). However, these methods are time-consuming as they can detect only a few

pathogens at a time and require large volumes of DNA for the detection of multiple pathogens. Furthermore, very little is known about the occurrence of endosymbionts of ticks from Pakistan which might also play a role in tick species survival and disease ecology in bovines (Karim et al., 2017). To address these issues, a novel microfluidic-based high-throughput method has been developed and used for epidemiological and surveillance studies in Europe and South Asia (Michelet et al., 2014; Cabezas-Cruz et al., 2019a; Sprong et al., 2019). This method uses a small volume of the nucleic acid to perform parallel real-time PCRs on 48.48 or 96.96 chips and can process up to 2304 or 9216 individual reactions, respectively (Liu et al., 2003; Michelet et al., 2014).

This study aimed to investigate molecular epidemiology and the prevalence of microorganisms and their co-infections in bovine ticks from five AEZs of Pakistan using a novel microfluidic-based high-throughput method.

4.3. Materials and methods

4.3.1. Tick collection, identification and DNA extraction

Based on physiography, climate, land use and soil type, Pakistan is divided into 10 AEZs (Khan, 2004). However, the fodder availability and climatic conditions mainly govern the type of livestock species kept across different AEZs. The bovine population is mainly distributed in two provinces (Punjab and Sindh) of Pakistan. Ticks (n = 774) were collected from clinically healthy cattle (n = 242) and water buffaloes (n = 200) from 30 villages located in six districts of Punjab and Sindh from September to November 2017. These districts are located in five different AEZs and include Bahawalpur (Sandy desert), Okara (Northern irrigated plain), Jhelum and Layyah (Arid; two districts were selected to cover diversity within this zone) districts in Punjab and Sukkur (Southern irrigated plain) and Thatta (Indus delta) districts in Sindh (Figure 4.1).

Tick specimens from each animal were stored in separate tubes containing 70% ethanol. Subsequently, each tick was morphologically characterised under a dissecting microscope (Olympus SZ40, Japan) using dichotomous keys (Walker et al., 2003; Barker and Walker, 2014). Following morphological identification, ticks of the same species from the same

animal were pooled in one tube. This resulted in a total of 234 tubes where 131 of those contained ticks from cattle whereas 103 were from buffaloes. DNA was extracted from one tick per tube as per the protocol described previously (Ghafar et al., 2020). Morphological characterisation of ticks was validated using PCR by amplifying cytochrome *c* oxidase subunit 1 (*cox*1) gene, 16S rRNA gene, and the second internal transcribed spacer and these results published previously (Ghafar et al., 2020).

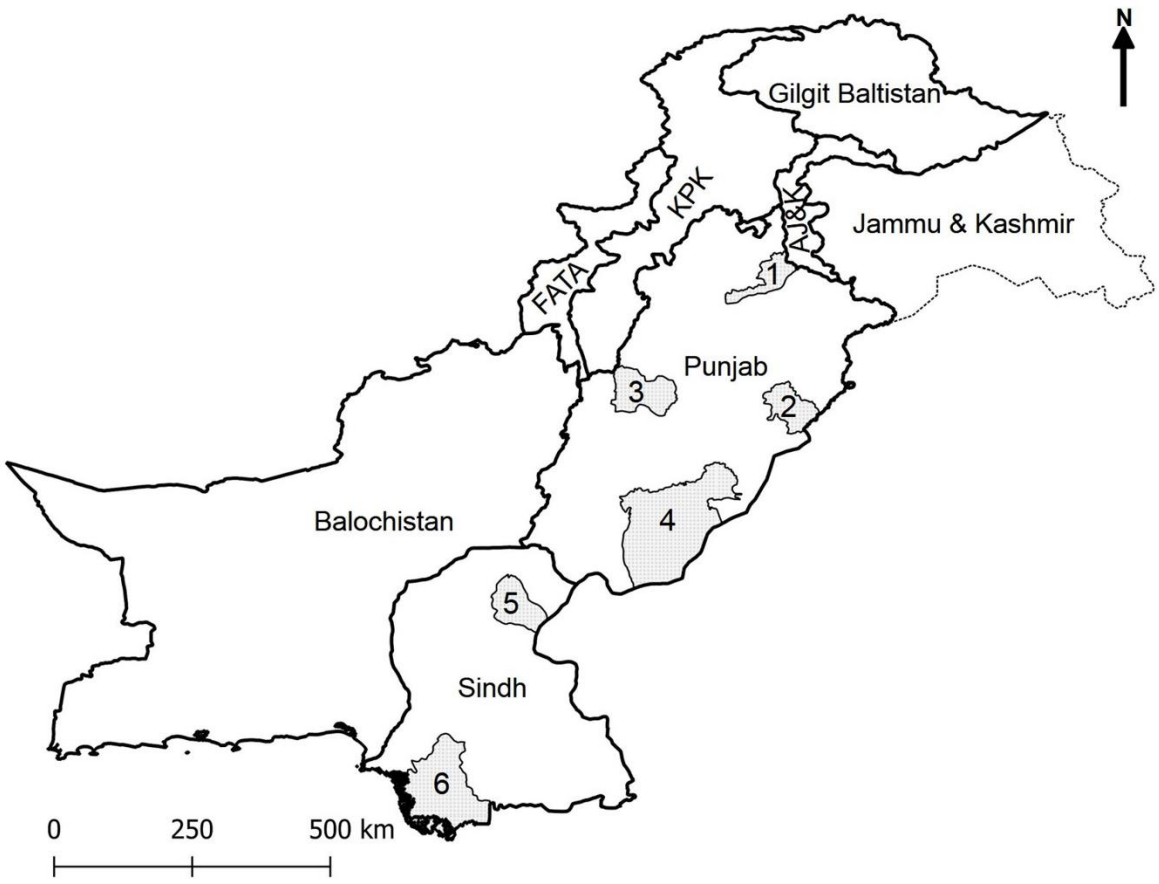

Figure 4.1. Map of Pakistan showing the districts (grey-coloured areas) included in this study. The names of districts include Jhelum (1), Okara (2), Layyah (3), Bahawalpur (4), Sukkur (5) and Thatta (6). Abbreviations: KPK, Khyber Pakhtunkhwa; FATA, Federally Administered Tribal Areas; AJ & K, Azad Jammu and Kashmir.

4.3.2. DNA pre-amplification

For DNA amplification, the Perfecta Preamp Supermix (Quanta Biosciences, Beverly, USA) was used according to the manufacturer's guidelines. Primers (targeted all microorganisms) were pooled combining equal volumes (200 nM final each), and the reaction was performed in a final volume of 5 µl containing 1 µl Perfecta Preamp 5×, 1.25 µl pooled primers mix, 1.5 µl distilled water and 1.25 µl DNA. PCR cycling conditions were one cycle at 95 °C for 2 min followed by 14 cycles at 95 °C for 10 s and 60 °C for 3 min. At the end of the cycling program, the reactions were diluted as 1:10 and the amplicons were stored at – 20 °C until further use.

4.3.3. Microfluidic real-time PCR

High-throughput microfluidic amplification was performed for major TBPs and potential endosymbionts using 48.48 dynamics array in a Bio-MarkTM real-time PCR system (Fluidigm, California, USA). These chips dispensed 48 samples and 48 PCR mixes into individual wells, followed by on-chip real-time PCR reactions in individual chambers and thermal cycling, resulting in 2,304 individual reactions. For more details regarding the development of this high-throughput tool based on real-time microfluidic PCRs (test of sensitivity, specificity, and controls used), please see Michelet et al. (2014).

Targeted microorganisms (and markers) were *Borrelia* spp. (23S), *Bo. burgdorferi* (*rpoB*), *Bo. garinii* (*rpoB*), *Bo. afzelii* (*fla*), *Bo. valaisiana* (*ospA*), *Bo. lusitaniae* (*rpoB*), *Bo. spielmanii* (*fla*), *Bo. bissettii* (*rpoB*), *Bo. miyamotoi* (*glpQ*), *Bo. mayonii* (*fla*), *Bo. bavariensis* (*pyrG*), *Anaplasma* spp. (16S), *A. marginale* (*msp*1), *A. platys* (*groEL*), *A. phagocytophilum* (*msp*2), *A. ovis* (*msp*4), *A. centrale* (*groEL*), *A. bovis* (*groEL*), *Ehrlichia* spp. (16S), *E. canis* (*gltA*), *Neorickettsia mikurensis* (*groEL*), *Rickettsia* spp. (*gltA*), *R. conorii* (ITS), *R. slovaca* (ITS), *R. massiliae* (ITS), *R. helvetica* (ITS), *R. aeschlimannii* (ITS), *R. felis* (*orfB*), *Bartonella* spp. (*ssrA*), *Ba. henselae* (*pap*31), *Francisella* spp. (*tul*4 and *fopA*), *Coxiella* spp. (*IS*1111 and *icd*), *Babesia microti* (*CCTeta*), *B. canis* (18S), *B. ovis* (18S), *B. bovis* (*CCTeta*), *B. caballi* (*rap*1), *Babesia* str. EU1 (18S), *B. divergens* (*hsp*70), *B. vulpes* (*cox*1), *Theileria* spp. (18S) and *Hepatozoon* spp. (18S). Briefly, amplifications were performed using 6-carboxyfluorescein (FAM)- and black hole quencher (BHQ1)-labelled TaqMan probes with

TaqMan Gene expression master mix as per manufacturer's recommendations (Applied Biosystems, Massachusetts, USA) (Michelet et al., 2014). PCR cycling conditions comprised of a denaturation step at 95 °C for 5 min followed by 45 cycles at 95 °C for 10 s, 60 °C for 15 s and 40 °C for 10 s. One negative control (water) was included per chip. Detection of ticks' 16S gene served as a positive control for the confirmation of DNA extraction. To assess PCR inhibitory molecules, present in tick DNA samples, DNA from *Escherichia coli* (EDL933 strain) was added to each sample as an internal inhibition control, and primers and probe specific for the *eae* gene of *E. coli* were used.

4.3.4. Validation of results by PCR and DNA sequencing

Microfluidic real-time PCR results were confirmed through conventional and nested PCR using different primers (Table 4.1) than those of the BioMarkTM system. Amplicons were sequenced by Eurofins MWG Operon (Ebersberg, Germany) and assembled using the Geneious Prime software (Biomatters Ltd, Auckland, New Zealand). An online BLAST (National Center for Biotechnology Information) was used to identify the sequenced organisms.

4.3.5. Phylogenetic analyses

The partial nucleotide sequences of microorganisms obtained for 18S rRNA, 16S rRNA and citrate synthase (*gltA*) genes were used to assess the genetic relationships with those of species of *Babesia*/*Theileria*, *Ehrlichia* and *Rickettsia*, respectively. Reference sequences were downloaded for each pathogen from GenBank and aligned separately (*Babesia*/*Theileria* over 582 bp; *Ehrlichia* over 618 bp; *Rickettsia* over 375 bp) with sequences obtained in this study, using ClustalW (Thompson et al., 1994) in the software MEGA v7.00 (Kumar et al., 2016). The best-fit evolutionary models for each dataset were selected based on Corrected Akaike's information criterion (cAIC) and Bayesian information criterion (BIC) using MEGA. Phylogenetic trees were constructed using the Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods in MEGA and Bayesian Inference (BI) method using Mr Bayes in Geneious Prime (Huelsenbeck and Ronquist, 2001). Each Bayesian analysis was run over 20,000,00 generations (ngen = 20,000,00) with two runs and

every 400th tree was saved (samplefreq = 400). For the NJ tree estimations, evolutionary distances were computed using the p-distance method whereas for the ML method, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. All positions containing gaps and missing data were eliminated. Bootstrapping method (10,000 replicates) was used to assess the reliability of internal branches and all trees were visualised and edited using MEGA. *Plasmodium falciparum* (GenBank: M19172), *A. marginale* (GenBank: AF414872) and *R. bellii* (GenBank: AY362703) were used as outgroups for *Babesia*/*Theileria, Ehrlichia* and *Rickettsia*, respectively.

4.3.6. Statistical analyses

Chi-square and Fisher's exact tests were used to evaluate the overall prevalence of microorganisms and to compare their prevalence in different AEZs. Data were analysed using GraphPad 5 Prism program (GraphPad Software Inc., La Jolla, CA, USA). The multiple correspondence analysis (MCA) was used to analyse the pattern of the association between infections (co-infections) and their distribution across provinces, districts and bovine host species. The inertia values were calculated by standard 'Burt matrix' method. The analyses were performed using the software, Statgraphics Centurion v. 16.1.03 (StatPoint Technologies Inc, Warrenton, VA).

Table 4.1. Primers used for validation of microfluidic real-time PCR results

Species	**Target gene**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Reference**
Babesia/*Theileria*	18S rRNA	GAGGTAGTGACAAGAAATAACAATA	TCTTCGATCCCCTAACTTTC	Gubbels et al 1999
Anaplasma/*Ehrlichia*	16S rRNA	GAACGAACGCTGGCGGCAAGC	AGTAYCGRACCAGATAGC CGC	Rar et al 2005
Rickettsia spp.	Citrate synthase	GGGGGCCTGCTCACGGCGG	ATTGCAAAAAGTACAGTGAAC	Regnery et al 1991
Hepatozoon spp.	18S rRNA	ATACATGAGCAAAATCTCAAC	CTTATTATTCCATGCTGCAG	de Azevedo et al 2016
Bartonella spp.	Citrate synthase	GGGGACCAGCTCATGGTGG	AATGCAAAAAGAACAGTAAACA	Norman et al 1995

4.4. Results

4.4.1 Diversity of microorganisms detected in ticks

PCR detected DNA of at least one microorganism in each of 221 ticks tested (94.4%, 221/234) (Figure 4.2; Table 4.2). *Francisella*-like endosymbionts (FLEs) were the most common (91.5%, 214/234) microorganisms detected in ticks (Table 4.2) followed by piroplasms (31.6%, 74/234), *Ehrlichia* spp. (20%, 47/234), *A. marginale* (7.7%, 18/234), *Borrelia* spp. (6.4%, 15/234), *A. centrale* (2.6%, 6/234), *Rickettsia* spp. (2.1%, 5/234), *A. ovis* (1.7%, 4/234), *R. massiliae* (1.7%, 4/234) and *Coxiella*-like endosymbionts (CLEs) (1.3%, 3/234). Furthermore, *A. phagocytophilum*, *Bartonella* spp., *Babesia ovis* and *Hepatozoon* spp. were also detected in 0.4% of ticks.

Overall, a high percentage of ticks was test-positive for DNA of microorganisms, with no significant variation ($\chi 2 = 10.0$, df = 5, $P = 0.075$) across districts (Sukkur and Layyah, 96%; Okara and Bahawalpur, 95%; Thatta, 93%; Jhelum, 87%) in different AEZs (Table 4.2). The prevalence of various pathogens (i.e. excluding endosymbionts) in ticks was high across districts (Bahawalpur, 60%; Thatta, 57%; Sukkur, 56%; Okara, 55%; Layyah, 50%; Jhelum, 33%) and it varied significantly ($\chi 2 = 19.2$, df = 5, $P = 0.0018$). The highest diversity of microorganisms was found in district Okara where DNA of eight of 14 (57.1%) microorganisms tested for was detected followed by Bahawalpur and Sukkur (7/14) and Layyah and Thatta (6/14) (Table 4.2). *Ehrlichia* spp., FLEs and piroplasms were found in all districts while *Bartonella* spp. and CLEs were only detected in Okara, *B. ovis* and *Hepatozoon* spp. in Layyah, and *R. massiliae* from Thatta. The prevalence of pathogens in ticks was significantly different between Jhelum and Bahawalpur ($P = 0.0002$), Jhelum and Thatta ($P = 0.0010$), Jhelum and Sukkur ($P = 0.0017$) Jhelum and Layyah ($P = 0.0214$) and Okara and Jhelum ($P = 0.0027$). At the provincial level, the diversity of microorganisms in ticks was higher in Punjab (11/14) than in Sindh (9/14) (Table 4.2). Based on the bovine host species, cattle ticks carried significantly higher piroplasms (41.2%, 54/131, $P = 0.0011$) than buffalo ticks (19.4%, 20/103); however, the overall prevalence of microorganisms in ticks did not vary significantly between both host species (Table 4.2).

Among four bovine tick species identified herein, the highest microorganism diversity (14/14) was found in *Hy. anatolicum*, with 96.2% (204/212) of them being test-positive for DNA of at least one microorganism, followed by *Rh. microplus* (4/14), *Hy. hussaini* (3/14) and *Rh. annulatus* (2/14) (Figure 4.3, Table 4.3). However, no microorganism was detected in the single specimen of *Hy. scupense* analysed (Table 4.3).

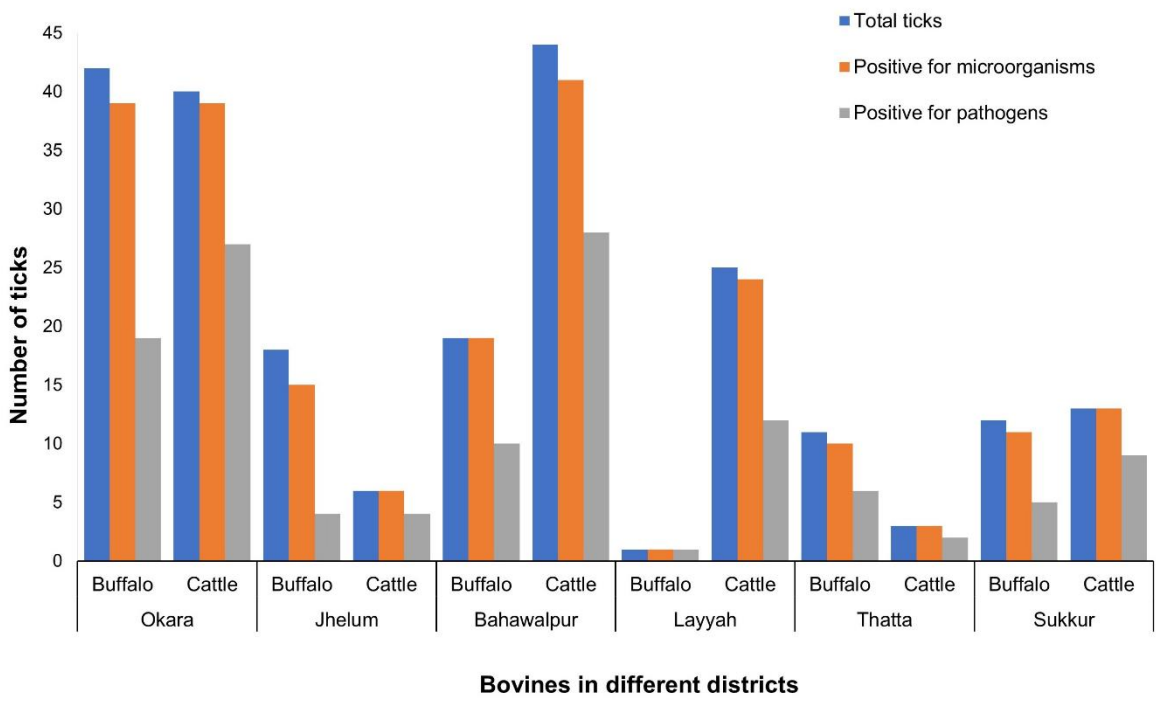

Figure 4.2. Information about microorganisms detected in ticks collected from bovines in different districts of two provinces, Punjab and Sindh, Pakistan

Table 4.2. Diversity of microorganisms in bovine ticks collected from six districts of Pakistan

Microorganisms	**Punjab**								**Sindh**				**Percentage positive (*n*/*N*)**
	Okara		Jhelum		Bahawalpur		Layyah		Thatta		Sukkur		
	B (*t*=42)	C (*t*=40)	B (*t*=18)	C (*t*=6)	B (*t*=19)	C (*t*=44)	B (*t*=1)	C (*t*=25)	B (*t*=11)	C (*t*=3)	B (*t*=12)	C (*t*=13)	
Borrelia spp.	3	3	2	–	1	4	–	–	–	–	1	1	6.4 (15/234)
Anaplasma marginale	4	8	–	2	2	1	–	–	–	–	1	–	7.7 (18/234)
A. phagocytophilum	–	–	–	–	–	–	–	–	1	–	–	–	0.4 (1/234)
A. ovis	–	–	–	–	1	2	–	–	–	–	–	1	1.7 (4/234)
A. centrale	–	3	–	–	1	1	–	1	–	–	–	–	2.6 (6/234)
Ehrlichia spp.	12	4	1	–	4	7	1	10	2	1	–	5	20 (47/234)
Rickettsia massiliae	–	–	–	–	–	–	–	–	3	1	–	–	1.7 (4/234)
Rickettsia spp.	–	–	–	–	–	–	–	–	3	1	1	–	2.1 (5/234)
Bartonella spp.	–	1	–	–	–	–	–	–	–	–	–	–	0.4 (1/234)
Francisella-like	39	39	14	5	19	40	1	23	7	3	11	13	91.5 (214/234)
Coxiella-like	2	1	–	–	–	–	–	–	–	–	–	–	1.3 (3/234)
Babesia ovis	–	–	–	–	–	–	–	1	–	–	–	–	0.4 (1/234)
Piroplasms	10	20	1	3	5	21	1	5	1		2	5	31.6 (74/234)
Hepatozoon spp.	–	–	–	–	–	–	–	1	–	–	–	–	0.4 (1/234)
Total	70	79	18	10	33	76	3	41	17	6	16	25	

B, Buffalo; C, Cattle; *n*, tested positive ticks; *N*, total tested ticks; *t*, total ticks tested from each bovine host per district

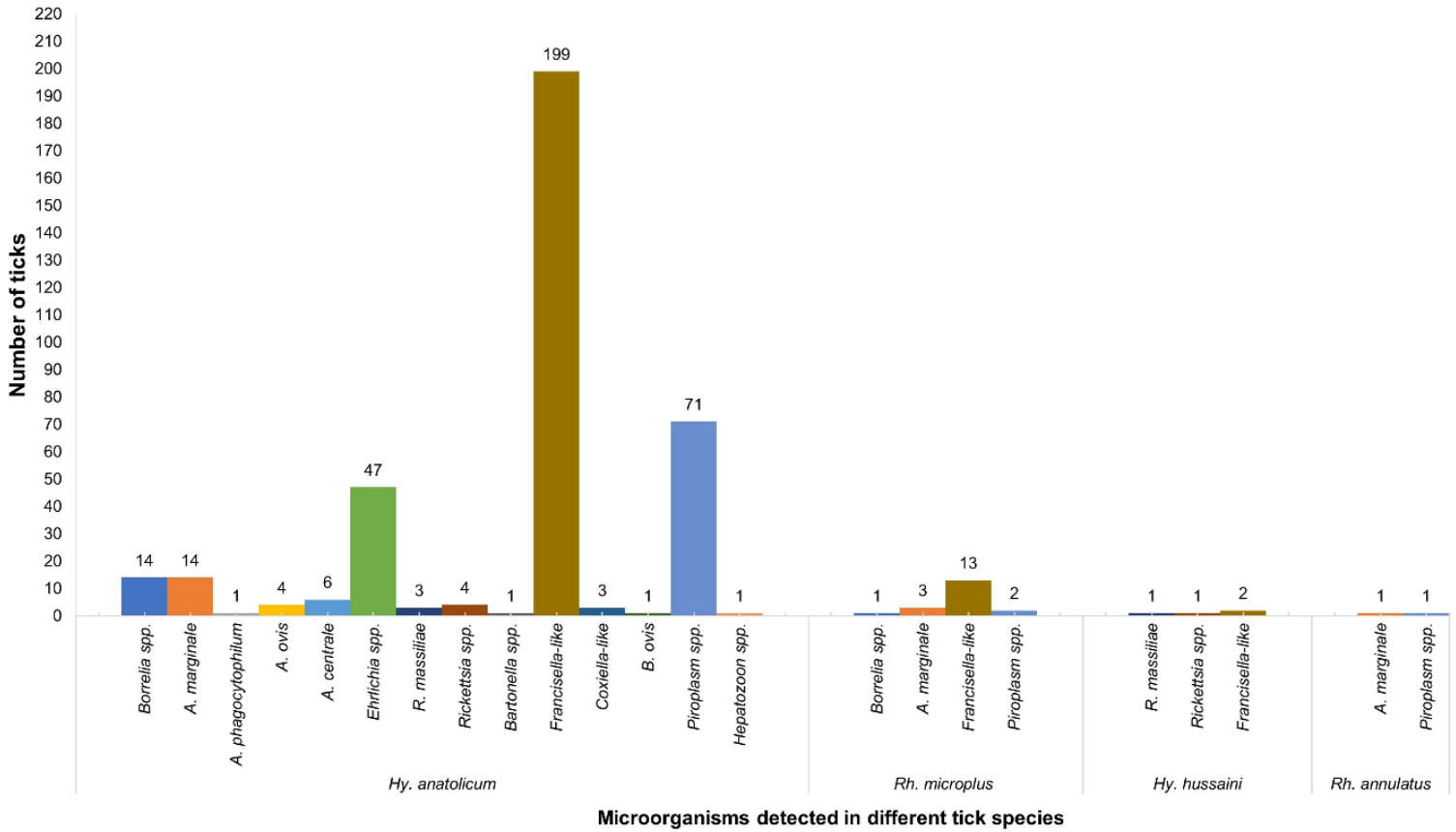

Figure 4.3. Information about microorganisms detected in different species of ticks collected from bovines in different districts of two provinces, Punjab and Sindh, Pakistan.

Table 4.3. Diversity of microorganisms in various tick species collected from cattle and buffaloes from the six districts of Pakistan

Tick species	**Study district**	**Bovine host**	**No. of ticks tested**	**No. of ticks infected**	**Detected microorganisms**
Hy. anatolicum	All six districts[a]	Buffalo	92	88	*Borrelia* spp., *A. marginale*, *A. phagocytophilum*, *A. ovis*, *A. centrale*, *Ehrlichia* spp., *R. massiliae*, *Rickettsia* spp., *Francisella*-like, *Coxiella*-like, piroplasms
		Cattle	120	116	*Borrelia* spp., *A. marginale*, *A. ovis*, *A. centrale*, *Ehrlichia* spp., *Bartonella* spp., *Francisella*-like, *Coxiella*-like, *B. ovis*, piroplasms, *Hepatozoon* spp.
Hy. hussaini	Thatta	Buffalo	2	1	*Francisella*-like
		Cattle	1	1	*Francisella*-like, *R. massiliae*, *Rickettsia* spp.
Hy. scupense	Jhelum	Buffalo	1	0	–
Rh. microplus	Okara and Jhelum	Buffalo	8	6	*Borrelia* spp., *Francisella*-like, piroplasms
		Cattle	9	8	*A. marginale*, *Francisella*-like, piroplasms
Rh. annulatus	Jhelum	Cattle	1	1	*A. marginale*, piroplasms
Total			234	221	

[a] Okara, Jhelum, Bahawalpur, Layyah, Sukkur and Thatta

4.4.2. Co-infections of microorganisms in ticks

Single infections with DNA of various microorganisms were present in 41% (96/234) of ticks, with FLEs (97.9%, 94/96) and piroplasm spp. (2.1%, 2/96) being the main microorganisms detected (Table 4.4). Among mixed infections of ticks, the highest percentage was found for double infections (36.8%, 86/234) followed by the triple (14.7%, 32/234), quadruple (2.1%, 5/234) and quintuple (0.9%, 2/234) infections. The most common double co-infection was with FLEs and piroplasm species (15.8%, 37/234) followed by FLEs and *Ehrlichia* spp. (9.4%, 22/234) whereas the most common triple co-infection was due to FLEs, piroplasm species and *Ehrlichia* spp. (6.8%, 16/234) (Table 4.4). *Hyalomma anatolicum* were positive for all types of single as well as mixed infections (i.e. single, double, triple, quadruple and quintuple) while the remaining tick species were positive for up to triple co-infections (Table 4.3). The distribution of tick infections with one or more microorganisms in various districts varied significantly, and ticks from Okara were positive for all five types of infections while those from Bahawalpur and Layyah had single, double, triple and quadruple infections. Ticks from Jhelum, Sukkur and Thatta were test-positive for DNA of one, two or three microorganisms only (Table 4.4).

Table 4.4. The occurrence of single and mixed infections of microorganisms in bovine ticks from six districts of Pakistan

Microorganisms	**Overall % prevalence**	**Proportion of ticks positive for microorganisms**					
		Okara (*n* = 82)[a]	Jhelum (*n* = 24)	Bahawalpur (*n* = 63)	Thatta (*n* = 14)	Sukkur (*n* = 25)	Layyah (*n* = 26)
Single infection							
Francisella-like	40.2	32	13	22	5	10	12
Piroplasms	0.9	0	1	1	0	0	0
Mixed infection with two microorganisms							
Francisella-like + Piroplasms	15.8	12	2	17	0	5	1
Francisella-like + *Ehrlichia* spp.	9.4	6	1	5	3	2	5
Francisella-like + *Borrelia* spp.	2.1	2	2	0	0	1	0
Francisella-like + *Rickettsia* spp.	0.4	0	0	0	0	1	0
Francisella-like + *A. marginale*	3.8	6	1	1	0	1	0
Francisella-like + *A. centrale*	0.9	1	0	1	0	0	0
Francisella-like + *Hepatozoon* spp.	0.4	0	0	0	0	0	1
Francisella-like + *Coxiella*-like	0.4	1	0	0	0	0	0
Ehrlichia spp. + Piroplasms	0.4	0	0	0	0	0	1
A. marginale + Piroplasms	0.4	0	1	0	0	0	0
Francisella-like + *A. ovis*	1.3	0	0	2	0	1	0
Rickettsia spp. + *R. massiliae*	1.3	0	0	0	3	0	0
Mixed infection with three microorganisms							
Francisella-like + Piroplasms + *Borrelia* spp.	1.7	2	0	2	0	0	0
Francisella-like + *Ehrlichia* spp. + *Borrelia* spp.	0.9	0	0	1	0	1	0
Francisella-like + *Ehrlichia* spp. + *A. centrale*	0.4	0	0	0	0	0	1
Francisella-like + Piroplasms + *A. phagocytophilum*	0.4	0	0	0	1	0	0
Francisella-like + *Ehrlichia* spp. + Piroplasms	6.8	7	0	4	0	2	3
Francisella-like + Piroplasms + *Coxiella*-like	0.4	1	0	0	0	0	0
Francisella-like + *Borrelia* spp. + *A. marginale*	0.4	0	0	1	0	0	0
Francisella-like + *A. marginale* + Piroplasms	1.3	3	0	0	0	0	0
Francisella-like + *A. ovis* + Piroplasms	0.4	0	0	1	0	0	0

Francisella-like + *A. centrale* + Piroplasms	0.4	0	0	1	0	0	0
Francisella-like + *R. massiliae* + *Rickettsia* spp.	0.4	0	0	0	1	0	0
Mixed infection with four microorganisms							
Francisella-like + *Borrelia* spp. + Piroplasms + *Ehrlichia* spp.	0.4	1	0	0	0	0	0
Francisella-like + *Borrelia* spp. + *Ehrlichia* spp. + *A. marginale*	0.4	0	0	1	0	0	0
Francisella-like + *A. marginale* + *A. centrale* + Piroplasms	0.4	1	0	0	0	0	0
Francisella-like + *Ehrlichia* spp. + *Coxiella*-like + Piroplasms	0.4	1	0	0	0	0	0
Francisella-like + *Ehrlichia* spp. + *B. ovis* + Piroplasms	0.4	0	0	0	0	0	1
Mixed infection with five microorganisms							
Francisella-like + *Borrelia* spp. + Piroplasms + *Ehrlichia* spp. + *A. marginale*	0.4	1	0	0	0	0	0
Francisella-like + *A. marginale* + *A. centrale* + *Bartonella* spp. + *Theileria* spp.	0.4	1	0	0	0	0	0
Total positives per district		78	21	60	13	24	25

[a] *Number of ticks examined in each district*

4.4.3. Genetic relationship of DNA sequences of selected microorganisms

Three different phylogenetic methods (BI, ML and NJ) were used to analyse (separately) genetic relationships of 16S rRNA gene sequences of *Ehrlichia* spp., 18S rRNA gene sequences of *Babesia* and *Theileria* spp. and *gltA* sequences of *Rickettsia* spp. with those of respective previously published sequences. The topologies of all three trees for each target organism were similar; hence, only NJ trees are provided herein (Figure 4.4-4.6). Two unique 16S rRNA sequences of *Ehrlichia* spp. (GenBank: MN726921 and MN726922) detected herein grouped with those previously published sequences from Thailand (GenBank: AF497581), Tibet, China (GenBank: AF414399) and Multan, Pakistan (GenBank: MH250197) (Figure 4.4), and had a 99.8–100% similarity to these three reference sequences. Two unique 18S rRNA gene sequences of piroplasms (GenBank: MN726546 and MN726547) were identified and one of these grouped with *T. annulata* sequences from Pakistan (GenBank: JQ743630) and Turkey (GenBank: MK918607), whereas the second sequence clustered with that of *B. occultans* from South Africa (GenBank: U09834) (Figure 4.5). For *Rickettsia* spp., only one unique *gltA* sequence (GenBank: MN728990) was found which grouped with *R. aeschlimannii* (GenBank: DQ235776) from Russia, *R. rhipicephali* (GenBank: U59721) from USA and *Rickettsia* sp. Bar (GenBank: U59720) from Spain (Figure 4.6).

4.4.4. Multiple correspondence analyses

Multiple correspondence analysis revealed no correlation between microorganisms and different districts, hosts or AEZs as microorganism clustered close to the centre of multidimensional matrices (Figure 4.7-4.9).

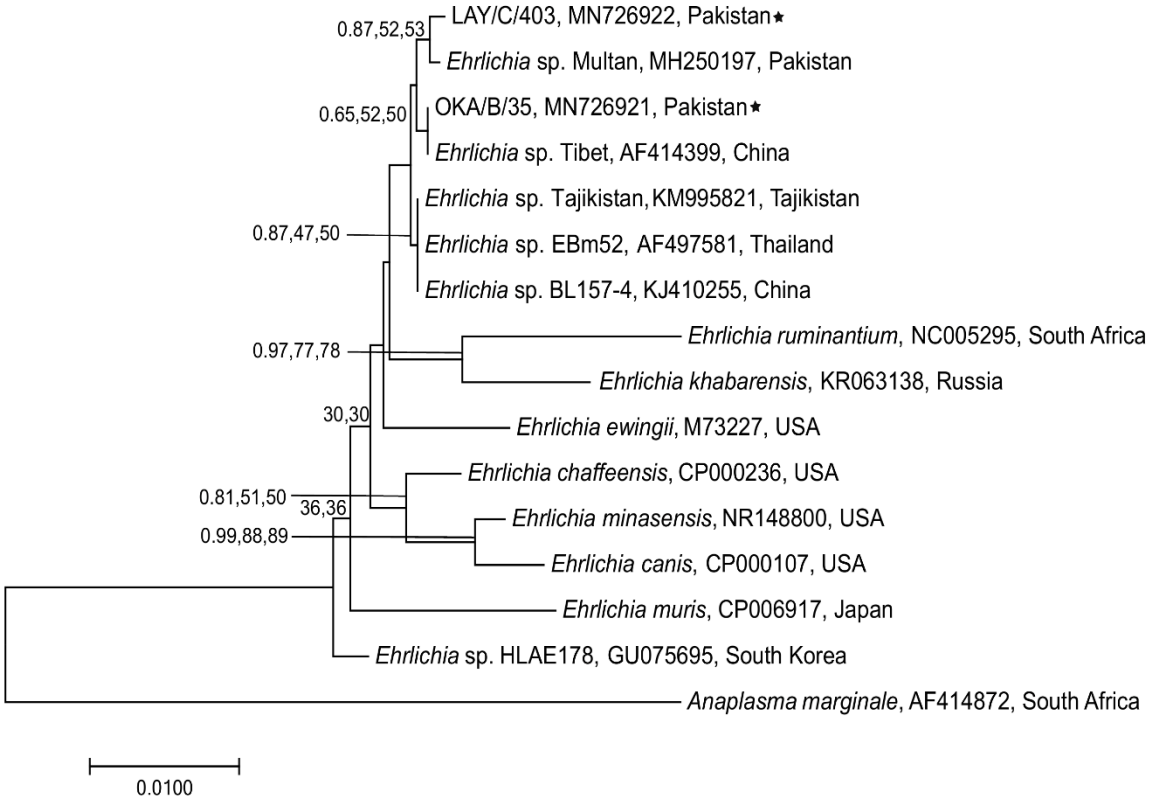

Figure 4.4. Genetic relationship of 16S rRNA gene sequences of *Ehrlichia* spp. identified in the present study (starred) with those of *Ehrlichia* spp. available on GenBank. The sequence data (618 bp) were analysed using Neighbour Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. There was a concordance among the topology of the BI, ML and NJ trees (not shown) and only NJ tree is presented here. Nodal support is given as a posterior probability of BI and bootstrap values for NJ and ML. The tree was rooted using *A. marginale* as outgroup. The scale-bar indicates the number of inferred substitutions per site.

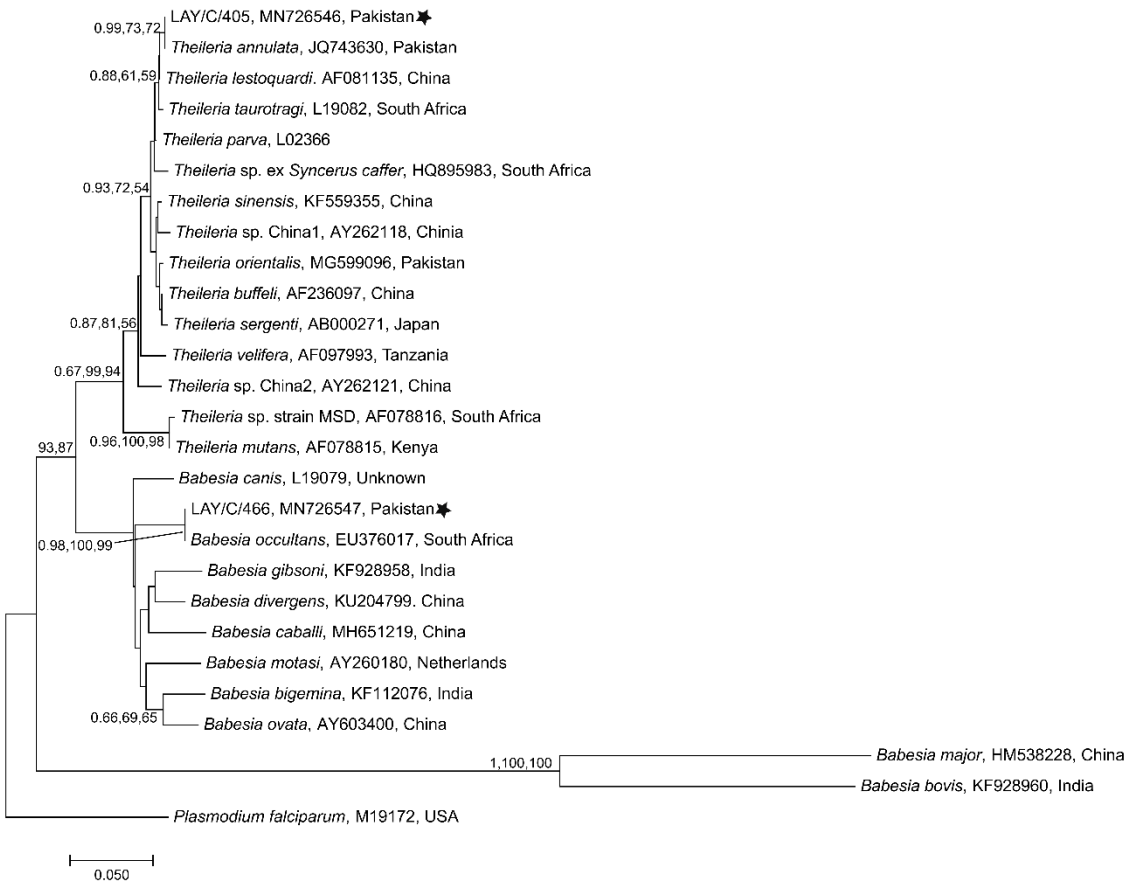

Figure 4.5. Genetic relationship of 18S rRNA gene sequences of *Babesia*/*Theileria* spp. identified in the present study (starred) with those of *Babesia*/*Theileria* spp. available on GenBank. The sequence data (582 bp) were analysed using Neighbour Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. There was a concordance among the topology of the BI, ML and NJ trees (not shown) and only NJ tree is presented here. Nodal support is given as a posterior probability of BI and bootstrap values for NJ and ML. The tree was rooted using *Plasmodium falciparum* as outgroup. The scale-bar indicates the number of inferred substitutions per site.

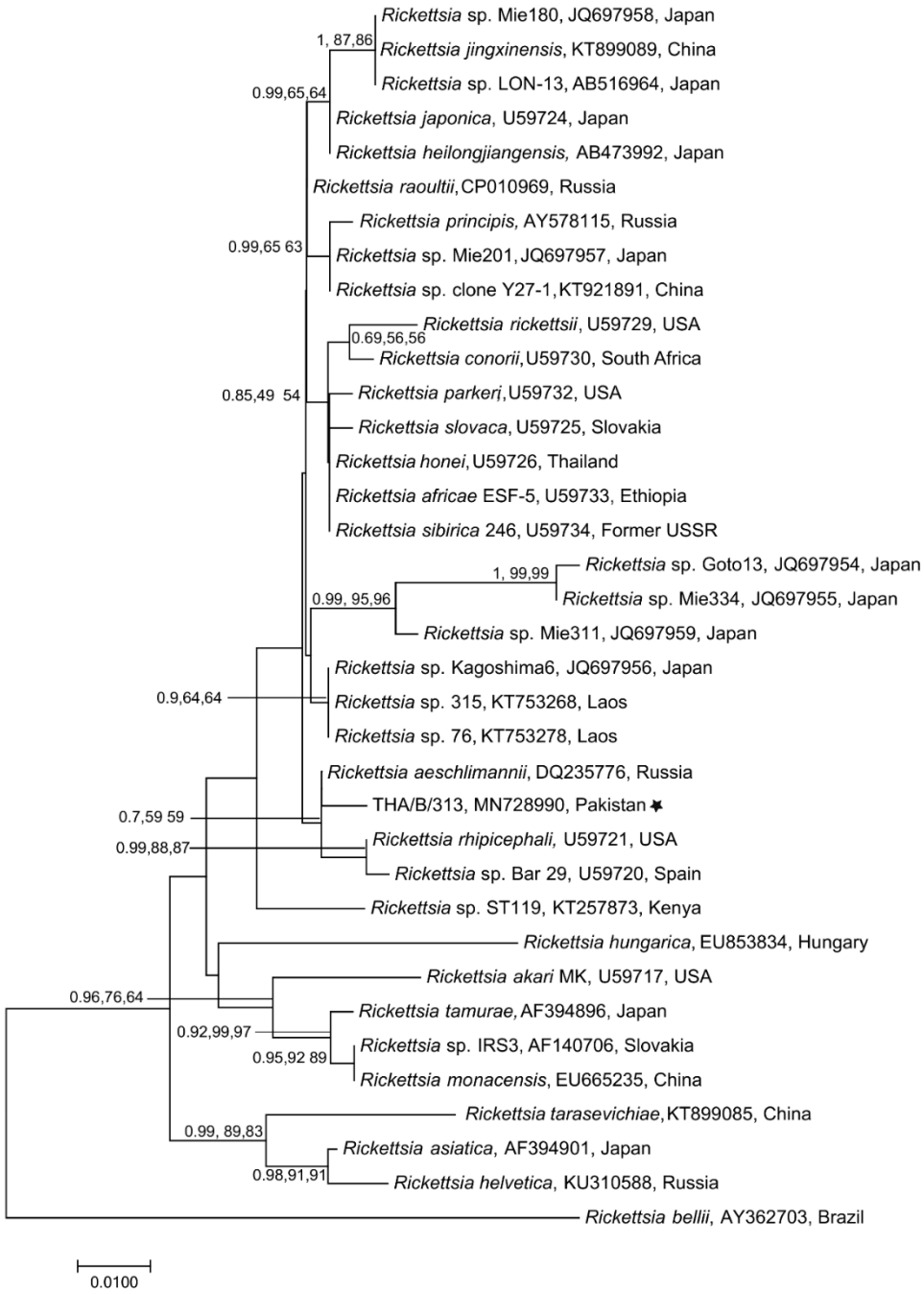

Figure 4.6. Genetic relationship of *gltA* sequences of *Rickettsia* spp. identified in the present study (starred) with those of *Rickettsia* spp. available on GenBank. The sequence data (375 bp) were analysed using Neighbour Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. There was a concordance among the topology of the BI, ML and NJ trees (not shown) and only NJ tree is presented here. Nodal support is given as a posterior probability of BI and bootstrap values for NJ and ML. The tree was rooted using *Rickettsia bellii* as outgroup. The scale-bar indicates the number of inferred substitutions per site.

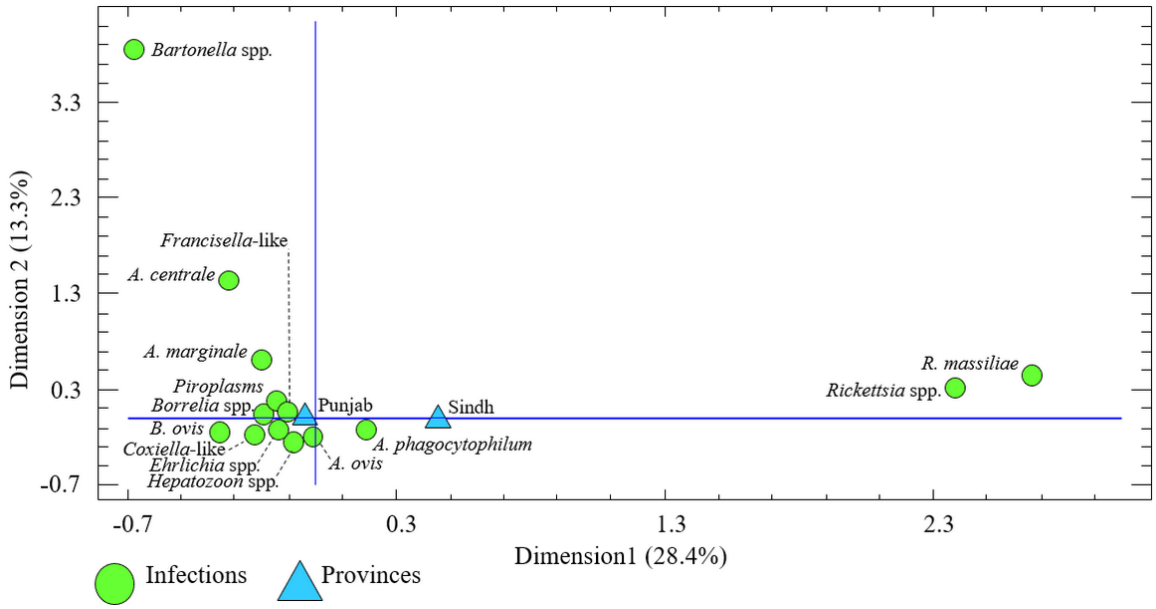

Figure 4.7. Multiple correspondence analyses maps from the projections of the first two dimensions, showing the associations between infections (co-occurrence), and their distributions among six districts in Punjab and Sindh provinces of Pakistan. Percentage in each dimension (axis) indicates the fraction of the inertia that each principal component explains. Analyses are shown for correlation between infections and their prevalence in different provinces.

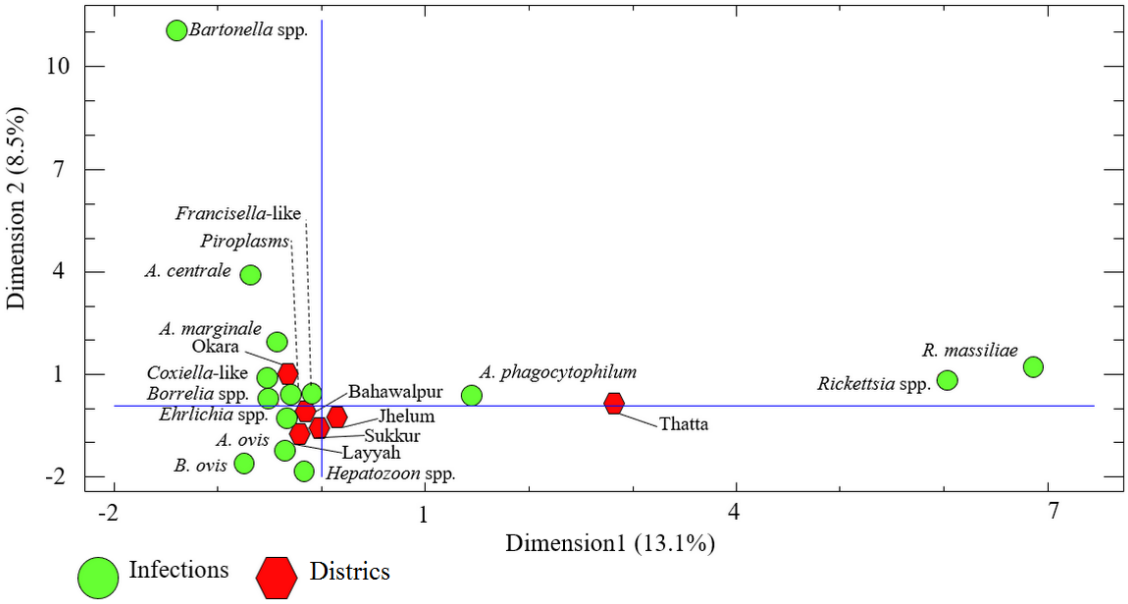

Figure 4.8. Multiple correspondence analyses maps from the projections of the first two dimensions, showing the associations between infections (co-occurrence), and their distributions among six districts in Punjab and Sindh provinces of Pakistan. Percentage in each dimension (axis) indicates the fraction of the inertia that each principal component explains. Analyses are shown for correlation between infections and their prevalence in different districts.

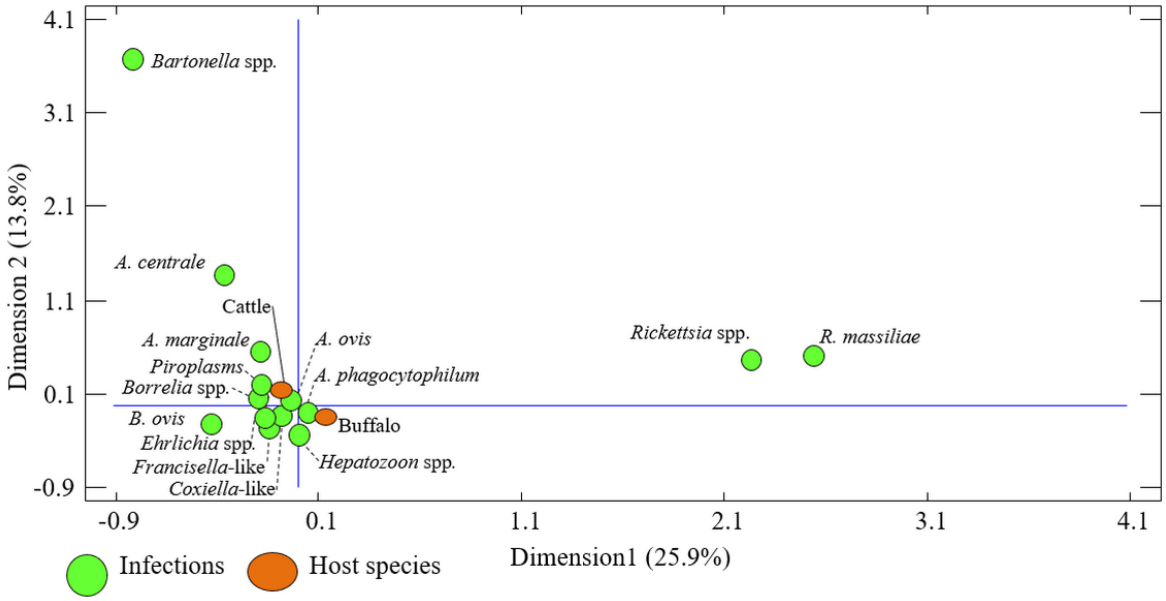

Figure 4.9. Multiple correspondence analyses maps from the projections of the first two dimensions, showing the associations between infections (co-occurrence), and their distributions among six districts in Punjab and Sindh provinces of Pakistan. Percentage in each dimension (axis) indicates the fraction of the inertia that each principal component explains. Analyses are shown for correlation between infections and their prevalence in bovine host species.

4.5. Discussion

This is the first study to utilise a high-throughput microfluidic technique for the detection of TBPs in bovine ticks collected from five AEZs of Pakistan. This real-time PCR technique offers a unique ability to detect multiple pathogens in ticks (Michelet et al., 2014; Cabezas-Cruz et al., 2019a; Sprong et al., 2019); here, we detected three protozoan genera (i.e. *Babesia*, *Hepatozoon* and *Theileria*) and 11 bacteria (species of *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella* and *Rickettsia*) of veterinary and/or public health significance. Interestingly, this study revealed that bovine ticks from Pakistan have (i) a diverse range of endosymbionts, with a higher prevalence of FLEs (91.5%); (ii) a high prevalence of TBPs (127 positives of 234 ticks tested); and (iii) a high frequency of co-infections (56.6% of total infections were co-infections). Due to low cycle threshold (Cq) values, genetic characterisation was not successful for some of the detected pathogens. As feeding ticks were collected from bovine hosts, we could not justify the bovine or tick origin of detected microorganisms. Likewise, this study does not argue the co-transmission of multiple microorganisms from a tick to the bovine host. However, such studies provide a snapshot of potential TBPs present in a region and help understanding disease(s) dynamics. Furthermore, all ticks tested in this study were collected during one season of the year, and therefore, this study does not inform about the seasonal variation of TBPs.

In this study, we detected the DNA of four *Anaplasma* spp. (*A. centrale, A. marginale, A. ovis* and *A. phagocytophilum*) in three species of ticks, *Hy. anatolicum, Rh. microplus* and *Rh. annulatus*. Bovine anaplasmosis is an endemic TBD in Pakistan and is mainly caused by *A. marginale* (Ashraf et al., 2013; Atif et al., 2013; Rehman et al., 2019). We found the highest prevalence of *A. marginale* in ticks from Okara district which could be attributed to the high prevalence of *Rh. microplus* in this region. Furthermore, this tick is the main vector for *A. marginale* (Kocan et al., 2010). This study reports the detection of *A. phagocytophilum* DNA in *Hy. anatolicum* for the first time from Pakistan. *Anaplasma phagocytophilum* is a zoonotic TBP infecting several mammalian species and can cause an acute febrile condition in humans, known as human granulocytic anaplasmosis (Dumler et al., 2001). In Pakistan, *A. phagocytophilum* is mainly responsible for equine granulocytic anaplasmosis and has been reported from equines (Saleem et al., 2018). Recently, *Anaplasma* species has been reported in bovines from Pakistan (Iqbal et al., 2019). Main tick vectors for the transmission of *A.*

phagocytophilum are *Ixodes* spp. but several other tick species have also been found positive for its DNA (Battilani et al., 2017). Additionally, *A. ovis* was detected in four specimens of *Hy. anatolicum* and it causes ovine anaplasmosis (Renneker et al., 2013; Shabana et al., 2018; Cabezas-Cruz et al., 2019b). Previously, *A. ovis* has been detected in small ruminants from Khyber Pakhtunkhwa (Talat et al., 2005) and in three tick species (*Hy. anatolicum*, *Hy. dromedarii* and *Rh. microplus*) from Punjab, Pakistan (Rehman et al., 2019). The detection of *A. ovis* DNA in multiple tick species indicates the challenges associated with multi-species livestock farming by small-holder dairy farmers.

We found the DNA of *Ehrlichia* species in 47 specimens of *Hy. anatolicum* from all six districts, with the highest percentage in Layyah (11/26) and lowest in Jhelum (1/24). Both districts are located within the same AEZ but hold a diverse topography because Jhelum district has plain and mountainous areas while Layyah district is comprised of the desert and plain lands. Furthermore, in Layyah, summer temperatures are higher, and the livestock population there is three times larger than in Jhelum, thereby possibly providing more suitable host and environmental factors for the growth of ticks and the transmission of TBPs. Sequence and phylogenetic analyses of 16S rRNA gene fragments of *Ehrlichia* spp. detected in this study showed a close similarity to those previously published from China [*Ehrlichia* sp. Tibet; (Wen et al., 2002)], Pakistan [*Ehrlichia* sp. Multan; (Rehman et al., 2019)] and Thailand [*Ehrlichia* sp. EBm52; (Parola et al., 2003)] (Figure 4.4). Although studies from Pakistan have reported ehrlichiosis in dogs using blood smear examination (Abbas et al., 2015) and molecular tools (Malik et al., 2018; Cabezas-Cruz et al., 2019a), no information is available on the epidemiology of ehrlichiosis in bovines from Pakistan. Recently, one study investigated TBPs in bovine ticks from Punjab, Pakistan, and reported a high prevalence of *Ehrlichia* spp. (21%) (Rehman et al., 2019). The high prevalence of *Ehrlichia* spp. in ticks reported in our study as well as in the previous study (Rehman et al., 2019) suggests that *Hyalomma* ticks might be transmitting ehrlichiosis in bovines in Pakistan; however, this hypothesis warrants further investigation.

A high number of piroplasms (*Theileria*/*Babesia* spp.; 31.6%) were detected in three tick species (i.e. *Hy. anatolicum, Rh. microplus* and *Rh. annulatus*). Microfluidic results were validated using piroplasm-specific primers targeting 18S rRNA gene and revealed that out of seven *Theileria*/*Babesia*-positive samples sequenced, five identical sequences belonged to

T. annulata while two remaining identical sequences were identified as *B. occultans*. Since sequencing was not undertaken for all samples, we were not able to determine individual prevalence rates for *Babesia* and *Theileria* spp. in ticks. *Babesia ovis* was also found in one tick sample using species-specific primers. All of these pathogens have been previously reported from Pakistan (Shahzad et al., 2013; Hassan et al., 2018a; Hassan et al., 2018b; Rehman et al., 2019). Theileriosis is an economically important disease affecting domestic and wild ruminants in tropical and subtropical regions of the world (Bishop et al., 2004). In Pakistan, this disease is mainly caused by the host cell-transforming sporozoan *T. annulata* and is responsible for high economic losses due to reduced production and mortalities, particularly in exotic and cross-bred animals (Jabbar et al., 2015). Additionally, *T. orientalis* has been reported in bovines and ticks from Pakistan (Ahmad et al., 2014; Hassan et al., 2018b) but clinical cases associated with this *Theileria* species complex still remain to be seen. Bovine babesiosis is another important TBD in Pakistan and is mainly caused by *B. bovis* and *B. bigemina* (Jabbar et al., 2015). However, we did not detect *B. bovis* DNA in any tick sample using species-specific primers and no tick was tested for *B. bigemina*, both of which were previously reported to occur in Pakistan (Karim et al., 2017; Hassan et al., 2018a; Rehman et al., 2019). However, during validation using *Babesia*/*Theileria* genera-specific primers, *B. occultans* was found in two *Hy. anatolicum* samples. Since its first detection in South Africa in 1981, *B. occultans* had been considered as apathogenic with its distribution limited to sub-Saharan countries (Gray and De Vos, 1981; Ros-García et al., 2012). However, recently it was associated with a clinical babesiosis outbreak in Italy (Decaro et al., 2013) and was also detected in canine blood from India (Mandal et al., 2014) and ticks from Pakistan (Rehman et al., 2019) and China (Sun et al., 2019). As climate change can impact the distribution and occurrence of vector-borne diseases (Caminade et al., 2019), *B. occultans* might become an important TBP for bovines in future. Furthermore, we found that ticks from cattle carried significantly higher piroplasms compared to those collected from buffaloes which might be due to the natural variation in the susceptibility of the hosts to different pathogens as buffaloes are known to be asymptomatic reservoirs of *Babesia* spp., which are pathogenic to cattle such as *B. bovis* (Benitez et al., 2018).

This study detected, for the first time, DNA of *Borrelia* in 15 tick specimens of *Hy. anatolicum* (n = 14) and *Rh. microplus* (n = 1) in Pakistan. However, none of the seven

species-specific primer pairs employed in this study could verify species identity, and therefore, the pathogenic and/or zoonotic potential of the detected *Borrelia* species could not be established. *Borrelia* species include spirochetes belonging to Lyme borreliosis and relapsing fever spirochete groups as well as intermittent clades and are transmitted by the body louse and hard and soft ticks (Barbour et al., 2017; Margos et al., 2018). There is no surveillance and/or diagnostic system in place for borreliosis in animals or humans in Pakistan; thereby, it remained unknown until now. Further testing is required to characterise *Borrelia* spp. and to assess their pathogenic and zoonotic potential.

We found that one *Hy. hussaini* and three *Hy. anatolicum* ticks contained DNA of *R. massiliae*, whereas, five ticks (one *Hy. hussaini* and four *Hy. anatolicum*) were positive for the DNA of unidentified *Rickettsia* spp. DNA sequencing followed by phylogenetic analyses revealed that *gltA* sequences of *Rickettsia* spp. determined herein clustered with those of *R. aeschlimannii* (Figure 4.6). This study provides the first report of *Rickettsia* spp. in bovine ticks from Sindh Province of Pakistan as they have previously been detected in ticks from Punjab (Robertson and Wisseman, 1973; Rehman et al., 2019), and Islamabad and Azad Jammu and Kashmir, Pakistan (Karim et al., 2017). *Rickettsia massiliae* and *R. aeschlimannii* belong to spotted fever group and both species are of public health significance (Vitale et al., 2006; Parola et al., 2013) as reported in the USA, Africa, Asia and Europe (de Mera et al., 2009; Yu et al., 2017). Overall, rickettsial infections rank second after Dengue in Southeast Asia as the cause of non-malarial febrile illnesses (Acestor et al., 2012). However, due to the lack of clinical testing facilities in Pakistan and other developing countries, these infections in animals and humans either remain unreported or underreported. We also found *Bartonella* spp. and *Hepatozoon* spp. in *Hy. anatolicum* ticks from Okara and Layyah districts, respectively. However, species identification of these organisms could not be established, and therefore, the pathogenic or zoonotic potential could not be assessed.

In this study, a high prevalence of endosymbionts such as FLEs (91.5%) was inferred, for the first time, in *Hy. anatolicum*, *Rh. microplus* and *Hy. hussaini* ticks from Pakistan. Previously, a study by Karim et al. (2017) investigated tick microbiomes of specimens collected from different livestock species from various parts of Pakistan and reported endosymbionts, *Francisella* (0.2% in *Hy. anatolicum* ticks from buffaloes) and *Coxiella* (7.9% in ticks belonging to the genera *Rhipicephalus*, *Haemaphysalis*, *Hyalomma* and

Ornithodoros) for the first time from Pakistan. Endosymbionts are non-pathogenic mutualistic and/or commensal microbes, and they are also abundant in ticks. Main tick endosymbionts belong to the genera of *Rickettsia*, *Francisella* and *Coxiella* (Ahantarig et al., 2013; Moutailler et al., 2016). Endosymbionts can (i) have multiple effects (detrimental or beneficial) on their carriers (Gray et al., 2009; Ahantarig et al., 2013; Guizzo et al., 2017); (ii) cause diseases to humans (Sanchez et al., 1992; Apperson et al., 2008); and (iii) interact with other TBPs and affect their colonisation and transmission (Macaluso et al., 2002; de la Fuente et al., 2003; Lively et al., 2005). The composition of endosymbionts can vary significantly among ticks in different parts of the world and it can be affected by several factors such as environment (Heise et al., 2010), season (Lalzar et al., 2012), geographical location (Van Overbeek et al., 2008), tick species (Lalzar et al., 2012), tick life stage (Moreno et al., 2006), feeding status (Heise et al., 2010) and co-existing pathogens (Abraham et al., 2017). Given that endosymbionts could play role in the prevalence and transmission of various pathogens, further investigations are required to explore the endosymbiotic communities in ticks infesting animals in Pakistan. Mixed infections with FLEs and piroplasm spp. and/or *Ehrlichia* spp. were most commonly detected in ticks across all AEZs. Ticks co-infected with multiple pathogens might pose a greater risk to animals as well as humans because of increased risk of co-infections which would ultimately increase the clinical complexity of diseases. High co-infection rates of microorganisms in ticks highlights the need of such studies to be conducted on larger populations to further assess pathogen communities and their potential interactions as well as their pathogenic or zoonotic potential.

4.6. Conclusions

This study reports that multiple TBPs of animal and public health significance are carried by bovine tick population in Pakistan. Co-infections with multiple pathogens are common, and endosymbionts are ubiquitously present. Overall, the prevalence of TBPs does not vary significantly across different AEZs of the country, but some pathogens may be restricted to particular regions or AEZs. Future studies are required to characterise endosymbionts further to explore their possible interaction(s) with pathogens transmitted by ticks collected from a larger animal population across different seasons in various AEZs.

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CHAPTER 5. GENERAL DISCUSSION

This thesis aimed to investigate: (i) major bovine health and production (BH&P) constraints, including ticks and tick-borne diseases (TTBDs) (Chapter 2); (ii) the epidemiology (i.e. prevalence and diversity) of bovine ticks (Chapter 3); and (iii) the epidemiology of tick-borne microorganisms in five agro-ecological zones (AEZs) of Pakistan using molecular genetic tools (Chapter 4). All of these aims were achieved. This Chapter discusses the findings and implications in a broader context and makes some recommendations about future research of vector-borne diseases (VBDs) that might extend this thesis.

5.1. Achievements and discussion in relation to current knowledge

5.1.1. Participatory epidemiological investigation identifies significant knowledge gaps and emphasises its utility to better disease control when combined with molecular-epidemiological studies.

The participatory epidemiological (PE) investigation (Chapter 2) conducted using multiple tools (i.e. simple ranking, pairwise ranking, constraint profiling and constraint impact matrix scoring) was the first comprehensive evaluation of the major BH&P constraints and associated knowledge of small-scale farmers and animal-health professionals (AHPs) in Pakistan. Results of ranking activities conducted with both participant groups demonstrated strong agreement on the list of major BH&P constraints, which varied slightly across different AEZs. These findings supported the results of the few available previous PE studies conducted in one (Hussain et al., 2005; Ali et al., 2006; Khan, 2010) or two (Ghaffar et al., 2007) districts of Pakistan, and also other countries where similar mixed-farming systems exist (Chatikobo et al., 2013; Hopker et al., 2018). The agreement on the list of constraints could be due to the global significance of transboundary animal diseases (TADs; such as foot-and-mouth disease and haemorrhagic septicaemia) and other production constraints (parasites, reproductive disorders and mastitis). Knowledge assessment activities

were useful in identifying farmers' knowledge gaps about risk factors and associated indirect losses of constraints. Identification of risk factors is a pre-requisite for diagnosis, treatment and control of veterinary and public health issues, as a lack of awareness in the community about these factors can result in risky practices (e.g., manual picking of ticks and inoculating saliva of FMD-affected to healthy animals) and increased probability of disease transmission (Kozukeev et al., 2006; Arif et al., 2017; Jin et al., 2017). Low literacy and inadequate adoption and inefficiency of extension services can be reasons for such knowledge gaps (Ali et al., 2019). Another interesting finding in Chapter 2 was the identification of a difference in the constraint-prioritisation criteria by farmers (prioritised constraints which cause high animal mortality) compared with AHPs (constraints with high associated costs and production losses). This difference could be partly attributed to a lack of farmers' knowledge of indirect losses, and by AHPs' poor disease surveillance and under-reporting of TADs (Akhtar and White, 2003; Limon et al., 2014). The under-reporting of TADs could be due to a fear of loss of local and export markets and promotion opportunities because of a failure of vaccination campaigns or a lack of financial and human resources (Yadav et al., 2020). Nevertheless, the difference in the constraint-prioritisation, if not addressed, could negatively impact the success rates of farmer education programs which are usually designed and executed by AHPs.

Findings of high prevalence of ticks and inefficiency of control methods reported by farmers and higher losses due to tick-borne diseases (TBDs) reported by AHPs were in agreement with the previous reports of high prevalence of TTBDs in Pakistan (Sajid et al., 2009; Jabbar et al., 2015; Rehman et al., 2017; Rashid et al., 2019; Rehman et al., 2019). Farmers' inability to relate ticks to any TBD of animals or humans could be partly due to lack of record-keeping, unawareness about tick-associated indirect losses or inadequate laboratory-based diagnostic facilities. Enhancing farmers' education about direct and indirect losses caused by ticks could contribute towards the control of TTBDs, as humans usually adopt better protective measures for constraints which have dire consequences. However, to design future education programs, further investigations of TTBDs using high-throughput molecular methods are required because of current knowledge gaps, including the diversity and seasonal variation in the prevalence and distribution of ticks and tick-borne pathogens (TTBPs); vector ecology and competence; endemic status of mammalian hosts; and efficacy

of available control measures (Jabbar et al., 2015). The knowledge gaps identified in Chapter 2 highlight the applicability of PE to animal health investigations and its significance for better disease control when combined with molecular-epidemiological studies.

5.1.2. A cross-sectional investigation of bovine ticks utilising classical and molecular methods proves invaluable to support the design of community education programs and TTBD control strategies.

The cross-sectional investigation of bovine ticks utilising morphological and molecular methods (Chapter 3) provided insights into the epidemiology of tick species in Pakistan. This was the first comprehensive characterisation of bovine tick species in five AEZs using multiple molecular markers. The finding of high tick infestation levels, both in cattle (47.9%) and in buffaloes (44%), confirmed the results of Chapter 2, in which ticks were found as a major BH&P constraint, according to both farmers as well as AHPs. These findings (Chapter 3) as well as previous reports of higher tick prevalence in bovines in Pakistan (Sajid et al., 2009; Rehman et al., 2017) contradict the concept of known tick resistance in buffalo and indigenous cattle (Horak et al., 2006; Benitez et al., 2012). The apparent high tick infestation levels of bovines emphasise that besides an expansion of tick populations due to climate change (Ogden and Lindsay, 2016) and selection of resistant tick populations (Abbas et al., 2014), bovine populations are also becoming more susceptible to tick infestation due to tick-host co-evolution (particularly buffaloes) in mixed farming systems (Mccoy et al., 2013) and an increased trend to crossbreed *Bos indicus* with *Bos taurus* to improve milk yield (Jongejan and Uilenberg, 2004; Hassan and Khan, 2013). A high prevalence (42.8%, 189/442) of *Hyalomma* (*Hy.*) *anatolicum* in both cattle (44.6%, 108/242) and buffaloes (40.5%, 81/200) across all AEZs in Pakistan indicated a broad habitat range and endemicity of this tick species. The success rate for species-level discrimination was 100% for mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) and 16S rRNA gene loci, but nuclear ribosomal internal transcribed spacer 2 (ITS-2) failed to distinguish between *Hy. anatolicum* and *Hy. excavatum*, a known limitation of ITS-2 locus for closely related species of ticks (Burger et al., 2014; Lv et al., 2014). Prior to genetic evidence of *Hy. hussaini* Sharif, 1928 in Chapter 3, only five partial nucleotide sequences (one for each 12S, 16S, 28S, ITS-2 and histone *H3*

regions) were available in the online databases for this tick species (Murrell et al., 2001; Sands et al., 2017). Interestingly, it had been widely known to infest domestic animals in India and Pakistan (Sharif, 1928; Neal et al., 1983), but there were rare reports of the occurrence of this tick species (Karim et al., 2017; Sands et al., 2017). This apparent decrease in the occurrence of *Hy. hussaini* could be attributed to a loss of habitat due to climate change, since ticks are no exception to becoming endangered or extinct (Mihalca et al., 2011; Léger et al., 2013; Dantas-Torres, 2015; Ogden and Lindsay, 2016). Further extensive molecular sampling from mammalian hosts and environment could assist in understanding the current status of this tick species. The usefulness of the molecular markers employed to identify different tick species indicated their utility for similar epidemiological investigations in other domestic as well as wild animals. Given the higher prevalence, broader habitat range (Chapter 3) and vector potential of *Hy. anatolicum* for a range of pathogens, the associated risks for animals and humans could be very high (Khurshid et al., 2015; Manjunathachar et al., 2019; Kasi et al., 2020). The public health significance of this tick species could be even higher in urban areas as depicted by the higher number of human infections with Crimean-Congo haemorrhagic fever (CCHF) near Eid-Ul-Azha (an Islamic festival) in Pakistan (Leblebicioglu et al., 2015; Atif et al., 2017). Molecular-genetic investigations of tick-borne pathogens (TBPs) within ticks and mammalian hosts would be useful to determine the potential threats as well as to design community education programs, and to develop control strategies for TTBDs.

5.1.3. The use of microfluidic real-time PCR assay sheds light on the microbiota of ticks and provides a powerful platform/tool for detailed and broader epidemiological explorations of microbiomes.

The use of microfluidic real-time PCR assay (Chapter 4) revealed single and multiple infections with 12 potentially pathogenic and two endosymbiotic microorganisms in ticks in different AEZs of Pakistan. A higher overall prevalence of pathogens (57.5%) in ticks indicated potential threats to mammalian hosts. Furthermore, the higher pathogen prevalence in ticks collected from cattle (65.1%) compared with those from buffaloes (47.4%) might relate to a higher level of innate resistance against TBPs in buffaloes than in cattle (Rajput et

al., 2005; Ponnudurai et al., 2017; Benitez et al., 2018; Larcombe et al., 2019). Although, several species of *Anaplasma*, *Ehrlichia*, endosymbionts and piroplasms found in Chapter 4 were already reported to occur in ticks from Pakistan (Jabbar et al., 2015; Karim et al., 2017; Hassan et al., 2018; Rehman et al., 2019), this study provided the first reports of *Anaplasma (A.) phagocytophilum*, *Bartonella*, *Borrelia* and *Hepatozoon* species. *Bartonella* infections are known in a wide range of mammals (Harms and Dehio, 2012), but are common in cats, *Bartonella* (*Ba*.) *henselae* being the causative agent of cat-scratch disease in humans (Harms and Dehio, 2012). In cattle, *Bartonella* infections cause endocarditis worldwide, and several clinical and experimental studies indirectly support the role of ticks as sources of infections (Angelakis et al., 2010; Telford III and Wormser, 2010; Erol et al., 2013; Gutiérrez et al., 2014; Ereqat et al., 2016). However, specific identification was not successful using the *Ba. henselae*-specific probes, indicating that the *Bartonella* taxa detected belonged to a different group. Similarly, a relatively high prevalence of *Borrelia* species and test-negative results for the 10 species-specific probes for *Borrelia* used in this study (Chapter 4) demand further molecular investigations to establish the significance and potential risks of what appear to be new taxa. *Hepatozoon* species, detected in a *Hy. anatolicum* tick in Chapter 4, are known to infect birds, reptiles and mammals, with feline and canine species being the most common hosts worldwide (Smith, 1996; Aktas, 2014; Wei et al., 2016; Kuo et al., 2018; Cabezas-Cruz et al., 2019). *Hepatozoon* infections have never been reported in cattle and *Hy. anatolicum* ticks infest a broader range of hosts, including dogs (Khan and Hussain, 2012). Therefore, it could be argued that the tick acquires *Hepatozoon* infections when feeding on dogs.

Since the first description of *Hy. hussaini* by Sharif (1928), Chapter 4 provides the first evidence of any microorganism (*Rickettsia* species and *Francisella*-like endosymbionts (FLEs) in this case) in this tick species. The prior lack of evidence for pathogens in this tick could be due to its limited distribution in the Indo-Pak region and rare reporting, but it is more likely due to the limited use of molecular methods for testing TBPs in the developing world. The ubiquitous presence of FLEs in *Hy. anatolicum* (93.9%) and *Rhipicephalus microplus* (76.5%) ticks was also a notable finding of Chapter 4, since these endosymbionts have been reported in tick species of genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* worldwide (Ivanov et al., 2011; Wójcik-Fatla et al., 2015; Azagi et al., 2017; Špitalská et al., 2018). Endosymbionts are known to affect the development (Guizzo et al., 2017) and the

vector potential of ticks (Narasimhan et al., 2014; Abraham et al., 2017); therefore, a better understanding of the microbiomes of ticks would be useful for the monitoring and surveillance of TTBDs. The detection of 14 microorganisms in *Hy. anatolicum* species (Chapter 4), higher prevalence and broader habitat range of this tick species in Pakistan (Chapter 3) and other Asian countries (Ghosh et al., 2007; Shyma et al., 2012) and its known zoonotic significance in CCHF transmission (Atkinson et al., 2013; Manjunathachar et al., 2019; Spengler et al., 2019; Kasi et al., 2020) implicates that this tick species is a risk to veterinary and public health in the region. Taken together, the findings show the benefits of using novel microfluidic assays in regional-scale investigations of VBDs in resource-scarce settings, where frequent or routine surveillance studies are not practicable. The finding of a high prevalence of pathogens in ticks indicates potential risks to mammalian populations, and a need for more profound molecular-epidemiological investigations of TBPs in these populations.

5.2. Implications and future research directions

This thesis demonstrates the advantages of combining molecular tools with traditional methods of tick identification and the usefulness of PE tools in animal health investigations in neglected communities and the importance of resulting data for designing community awareness and disease control programs. This thesis also highlights the significance of using a novel microfluidic real-time PCR for rapid detection of tick-borne microorganisms in regional-scale epidemiological investigations in underprivileged settings like Pakistan, where molecular-epidemiological data are scarce. Moreover, it identifies important areas for future investigation, including tick vector competence, and the epidemiology, ecology, and genomics of TTBPs, tick-host-microbiome interactions, and sero-epidemiology of TBPs for the control of TTBDs.

A systematic investigation of bovine TTBDs in five AEZs of Pakistan gained information on major BH&P constraints including TTBDs (Chapter 2), epidemiology of tick species (Chapter 3) and the tick-borne microorganisms (Chapter 4). To date, questionnaire-based surveys are the most common data collection tools, despite several drawbacks (Ponto, 2015). The use of PE has been predominantly limited to livestock-related investigations in Africa

and, to a lesser extent, in Asia (Allepuz et al., 2017). However, PE has recently emerged as a major public health surveillance system in the form of community-based participatory research (Brownstein et al., 2017; Holliday et al., 2020). Besides this, PE has broader applications including, but not limited to, investigations of healthcare perceptions (Hulen et al., 2019; Kjellström and Mitchell, 2019; Leh and Saoud, 2019), women health (Garbers et al., 2020), mental health (Thompson et al., 2020) and zoonotic diseases (Arif et al., 2017; Yano et al., 2018; Ebata et al., 2020). Additionally, PE has also been utilised to investigate various aspects of TTBDs; including the prevalence and distribution (Okuthe and Buyu, 2006; Byaruhanga et al., 2015a; Lieske and Lloyd, 2018; Nieto et al., 2018), impact (Adane et al., 2012; Boucher et al., 2019), management and control (Ocaido et al., 2004; Wanzala, 2012; Byaruhanga et al., 2015b; Byaruhanga et al., 2015c) as well as related knowledge within communities (Chenyambuga et al., 2010; Sungirai et al., 2016; Kimaro et al., 2017), particularly in Africa. Recently, the use of mobile phone-based PE tools for disseminating and collecting information during the COVID-19 pandemic (Garg et al., 2020), disaster preparedness and recovery (Mercer et al., 2008) and TTBDs (Chepkwony et al., 2018; Porter et al., 2019; Chauhan et al., 2020) have further highlighted the significance of PE. A similar framework (such as mobile phone-based applications) could be adopted for assessing knowledge, risk factors, incidence, prevalence and control practices in vector-borne disease investigations, particularly in communities where face-to-face interactions are not possible due to time restraints, limited finances and/or logistic or cultural issues.

In recent years, novel molecular methods have been developed for VBD diagnosis and surveillance and microfluidic real-time PCR is one such tool. This tool has the capacity of running up to 9216 individual PCRs, allowing detection of 96 targets in 96 samples per run (Michelet et al., 2014) and, thus, providing a rapid snapshot of microbial populations, as also demonstrated in this study (Chapter 4). It has been employed successfully in epidemiological investigations of symbionts and pathogenic species of bacterial, parasitic and viral origin in ticks of livestock and companion animals as well as exploring co-infections, tick-pathogen interactions, transmission mechanisms and pathogen locations within tick organs (Michelet et al., 2014; Gondard et al., 2018; Lejal et al., 2019; Gondard et al., 2020a; Grech-Angelini et al., 2020). This tool could be particularly applicable to regional-scale epidemiological investigations of TBPs of livestock and other animals in the developing world, where only a

few epidemiological data are available. Furthermore, recent innovations of paper-based microfluidics for malaria diagnosis (Reboud et al., 2019) and smartphone-based platforms for detection of Chikungunya, Dengue, and Zika viruses (Ganguli et al., 2017; Ong and Poljak, 2020) could be adopted for field diagnosis and surveillance of major TBPs, particularly in low-resource communities where laboratory facilities are scarce.

Despite the high prevalence and significant impact of TTBDs on farmers' livelihoods, little is known about the seasonal epidemiology of these diseases in different farming systems of Pakistan. Tropical theileriosis, babesiosis and anaplasmosis are major bovine TBDs in Pakistan (Jabbar et al., 2015) and the results of this study (Chapter 4) also demonstrated a high prevalence of piroplasms (*Babesia*/*Theileria*) and *Anaplasma* species in tick populations. Therefore, longitudinal epidemiological investigations inclusive of different farming systems and seasons would significantly address the existing knowledge gaps for control of TTBDs in this country. Traditionally, the microscopic examination of blood smears has been the "gold standard" for diagnosis of haemoparasites worldwide (Moody and Chiodini, 2000). However, this technique often fails to detect carrier status known to occur in *Anaplasma*, *Babesia* and *Theileria* infections (Robinson, 1982; Pipano and Shkap, 2000; Lew-Tabor, 2016; Alvarez et al., 2019). Similar limitations, such as low sensitivity and specificity, and a failure to detect chronic infections, are also associated with the use of serological assays (Wagner et al., 1992; d'Oliveira et al., 1995). These limitations have been overcome with the advent of molecular tools with high throughput capacity, such as next-generation sequencing (NGS). NGS is now a routine part of biological research, has several advantages over conventional diagnostic tools, including higher sensitivity and specificity, and an ability to detect mutations and sequence variants by massively parallel sequencing. Additionally, the combined use of NGS and bioinformatic tools should allow the tracking of geographical origin, spread and pathogen sources in temporal and spatial studies (Maljkovic et al., 2020). However, NGS also generates millions of reads of host DNA, which create a background and need to be filtered from the final dataset for subsequent analysis. This problem can be resolved through the amplification of one or more particular regions of the genome and subsequent massive parallel sequencing (Johnsen et al., 2013; Anis et al., 2018). This approach is referred to as targeted-NGS and has been applied to eukaryotes (Chaudhry et al., 2019; Glidden et al., 2019; Kounosu et al., 2019; Nguyen et al., 2020), prokaryotes

(Egan et al., 2020) and viruses (Gondard et al., 2020b). Nowadays, NGS is also being used for understanding the complex phylogenies of TTBPs through phylogenomics (Mans et al., 2016; Liu et al., 2018; Barbosa et al., 2019; Charrier et al., 2019; Young and Gillung, 2019). Its application to the microbiota in/on mammal groups and vector populations would be central to unravelling aspects of the epidemiology, population genomics and diversity of TBDs.

Another important aspect identified in Chapter 2 was a lack of effective methods to control TTBDs in Pakistan and worldwide. The control of TTBDs worldwide relies primarily on the use of acaricides (Estrada-Peña et al., 2020; Wilson et al., 2020). However, widespread acaricide-resistant tick populations, and environmental contamination and toxic residues of acaricides in milk and meat are some of the reasons to seek alternatives to chemotherapeutic intervention (George, 2000). Therefore, there is a need for developing highly effective and eco-friendly approaches for the sustained prevention and control of TBDs (de la Fuente et al., 2017). Resistance development can be slowed through a strategic or integrated use of acaricides, but need to be monitored, also supported by in-vitro and field trials of acaricides as well as ongoing epidemiological investigations of prevalence and distribution of tick species and the effects of host- and climate on tick populations. There is need to investigate the level of acaricide resistance in ticks since it has been reported from neighbouring countries including India but no study has assessed it in Pakistan (Kumar et al., 2020; Upadhaya et al., 2020). Educating farmers would be critical to guide them to use of acaricides in an effective and sustainable way and PE tools could be useful for guiding the development and execution of such education programs.

Omics-derived tick microbiome information could assist developing new methods of TTBD control by providing insights into symbionts known to affect the vector development and competence (Coon et al., 2014; Dennison et al., 2014). For instance, it has been proposed that transmission of tick-borne pathogens (e.g., *Ehrlichia chaffeensis*, *A. marginale* and *Borrelia burgdorferi*) are altered by other symbionts (Burgdorfer et al., 1973; Klyachko et al., 2007; Narasimhan et al., 2014; Gall et al., 2016). This concept has been best-demonstrated by *Wolbachia* being able to prevent mosquito-borne human diseases, including Chikungunya, Dengue, and malaria (Moreira et al., 2009; Bian et al., 2010). Further, the recently-identified role of *Microsporidia MB* in the transmission blockage of *Plasmodium*

falciparum in *Anopheles arabiensis* mosquitoes could be a major breakthrough in malaria control (Herren et al., 2020). The use of recombinant microbes for reducing vector competence (Hurwitz et al., 2011; McClure et al., 2017) and *Metarhizium* spp. fungi for biological control (Aw and Hue, 2017) of ticks appear to offer promise for the integrated control of TBDs. Additionally, the more recently-developed CRISPR/Cas9-based method, called receptor-mediated ovary transduction of cargo (ReMOT-control) for the genetic manipulation of mosquitoes (Chaverra-Rodriguez et al., 2018; Macias et al., 2020) and other vectors including mites (Dermauw et al., 2020), has opened up new avenues for the control of VBDs. However, several important research areas of TTBPs are poorly explored, particularly in under-developed communities. These include tick ecology, microbiomes, interactions among ticks, host affiliations, pathogens and climate, the efficacy of available control strategies and mathematical modelling of the prevalence and transmission of TTBPs (de la Fuente et al., 2020; Wilson et al., 2020). Furthermore, there is a need to utilise the concept of endemic stability for the control of *Theileria*, *Babesia* and *Anaplasma* infections and the diseases that they cause (Darghouth et al., 1996; Regassa et al., 2003; Heuer et al., 2004; Gachohi et al., 2010), as well as for other diseases of public health significance, such as malaria (Coleman et al., 2001). The endemic stability is established for TBPs if more than 75% of the mammalian host population is exposed to infections before reaching nine months of age (Mahoney, 1974; Mahoney et al., 1981; Goff et al., 2001; Jonsson et al., 2012). Although the successful use of this concept requires the continuous collection and analysis of data on the seroprevalence of TBPs, tick infestation and pathogen infection rates (Jonsson et al., 2012), cross-sectional seroprevalence studies in young calves might provide a means of estimating endemic stability in a population (Regassa et al., 2003; Gachohi et al., 2010; Martins et al., 2010). Taken together, future TTBDs control would ideally be achieved through rational use of acaricides and/or vaccines based on both tick- and pathogen-derived antigens coupled with use of novel gene-editing techniques for ticks and implementation of breeding strategies for improved host resistance (de la Fuente, 2018; Burrow et al., 2019; de la Fuente et al., 2020; Estrada-Peña et al., 2020).

5.3. Conclusions

This thesis has gained important new knowledge about TTBDs of bovines in Pakistan and highlights the significance of using molecular-epidemiological tools for the surveillance of TTBDs. It has also shed light on the benefits of utilising tools of participatory epidemiology in animal-health investigations. Overall, the findings of this thesis demonstrate a high prevalence of TTBDs in Pakistan and emphasise the need for further intensive research on the epidemiology, ecology and population genomics of TTBPs. The use of advanced tools will be critical to attaining an improved understanding of interactions among vectors, microbiomes, mammalian hosts and the environment, and should guide the development of an integrated strategy for the control of TTBDs in Pakistan and other countries around the world.

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APPENDIX

Supplementary File S1

Data S2.1

R Code for Regression Model

```
##Download required libraries##
library(ordinal) # for ordinal logistic models
library(RVAideMemoire) # easy way to get model predictions for ordinal model
library(emmeans) # also needed for model predictions
library(lattice) # for graphic
library(ggplot2)
library(RColorBrewer)
##prepare data files##
fpra <- read.csv("Supplementary_File_S2.csv")
table(fpra$dis)
disfreq <- table(fpra$dis)
dis.sel <- rownames(disfreq)[disfreq > 1]
fpra <- fpra[fpra$dis %in% dis.sel,]
fpra$dis <- factor(fpra$dis)
table(fpra$dis)
fpra$adis[fpra$dis == "Black quarter"] <- "BQU"
fpra$adis[fpra$dis == "FMD"] <- "FMD"
fpra$adis[fpra$dis == "Haemorrhagic septicaemia"] <- "HSE"
fpra$adis[fpra$dis == "Internal parasitism"] <- "IPA"
fpra$adis[fpra$dis == "Mastitis"] <- "MAS"
fpra$adis[fpra$dis == "Red water"] <- "RWA"
fpra$adis[fpra$dis == "Reproductive disorders"] <- "REP"
fpra$adis[fpra$dis == "Ticks"] <- "TIC"
fpra$milk <- factor(fpra$milk)
fpra$meat <- factor(fpra$meat)
fpra$cost <- factor(fpra$cost)
fpra$morbidity <- factor(fpra$morb)
fpra$mortality <- factor(fpra$mort)
# ------------------------------------------------------------------------------------------------------------
ahpr <- read.csv("Supplementary_File_S3.csv")
table(ahpr$dis)
disfreq <- table(ahpr$dis)
dis.sel <- rownames(disfreq)[disfreq > 1]
ahpr <- ahpr[ahpr$dis %in% dis.sel,]
ahpr$dis <- factor(ahpr$dis)
table(ahpr$dis)
ahpr$adis[ahpr$dis == "Black quarter"] <- "BQU"
```

```
ahpr$adis[ahpr$dis == "Blood parasites"] <- "BPA"
ahpr$adis[ahpr$dis == "FMD"] <- "FMD"
ahpr$adis[ahpr$dis == "Haemorrhagic septicaemia"] <- "HSE"
ahpr$adis[ahpr$dis == "Internal parasitism"] <- "IPA"
ahpr$adis[ahpr$dis == "Mastitis"] <- "MAS"
ahpr$adis[ahpr$dis == "Red water"] <- "RWA"
ahpr$adis[ahpr$dis == "Reproductive disorders"] <- "REP"
ahpr$adis[ahpr$dis == "Ticks"] <- "TIC"
ahpr$milk <- factor(ahpr$milk)
ahpr$meat <- factor(ahpr$meat)
ahpr$cost <- factor(ahpr$cost)
ahpr$morbidity <- factor(ahpr$morb)
ahpr$mortality <- factor(ahpr$mort)
#================================================================
# Milk probability calculation
fpr.clmilk <- clm(milk ~ adis, data = fpra)
# Model-based probabilities:
emilk <- emmeans(fpr.clmilk, ~ adis | cut, mode = "linear.predictor")
predmilk <- rating.emmeans(emilk, type = "prob")
head(predmilk)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmilk[predmilk$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmilk with xpos:
predmilk$xpos <- sort$xpos[match(predmilk$adis, sort$adis)]
predmilk$Rating <- factor(predmilk$Rating, levels = 0:6)
predmilk$xpos <- factor(predmilk$xpos, levels = sort$xpos, labels = sort$adis)
mypalette <- rev(c(rev(brewer.pal(6, "Greens")), "transparent"))
# Stacked bar chart using ggplot2:
fpr.milk <- ggplot(data = predmilk, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on milk") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); fpr.milk
#-----------------------------------------------------------------------------------------------------
ahp.clm <- clm(milk ~ adis, data = ahpr)
```

```
# Model-based probabilities:
emilk <- emmeans(ahp.clm, ~ adis | cut, mode = "linear.predictor")
predmilk <- rating.emmeans(emilk, type = "prob")
head(predmilk)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmilk[predmilk$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmilk with xpos:
predmilk$xpos <- sort$xpos[match(predmilk$adis, sort$adis)]
predmilk$xpos <- factor(predmilk$xpos, levels = sort$xpos, labels = sort$adis)
predmilk$Rating <- factor(predmilk$Rating, levels = 0:7)
mypalette <- rev(c(rev(brewer.pal(7, "Reds")), "transparent"))
# Stacked bar chart using ggplot2:
ahp.milk <- ggplot(data = predmilk, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on milk") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); ahp.milk
#-------------------------------------------------------------------------------------------------------
# Meat probability calculation
fpr.clmeat <- clm(meat ~ adis, data = fpra)
# Model-based probabilities:
emeat <- emmeans(fpr.clmeat, ~ adis | cut, mode = "linear.predictor")
predmeat <- rating.emmeans(emeat, type = "prob")
head(predmeat)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmeat[predmeat$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmeat with xpos:
predmeat$xpos <- sort$xpos[match(predmeat$adis, sort$adis)]
predmeat$Rating <- factor(predmeat$Rating, levels = 0:7)
predmeat$xpos <- factor(predmeat$xpos, levels = sort$xpos, labels = sort$adis)
mypalette <- rev(c(rev(brewer.pal(7, "Greens")), "transparent"))
```

```
# Stacked bar chart using ggplot2:
fpr.meat <- ggplot(data = predmeat, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on meat") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); fpr.meat
#-------------------------------------------------------------------------------------------------------
ahp.clmeat <- clm(meat ~ adis, data = ahpr)
# Model-based probabilities:
ahpmeat <- emmeans(ahp.clmeat, ~ adis | cut, mode = "linear.predictor")
predmeat <- rating.emmeans(ahpmeat, type = "prob")
head(predmeat)

# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmeat[predmeat$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmeat with xpos:
predmeat$xpos <- sort$xpos[match(predmeat$adis, sort$adis)]
predmeat$xpos <- factor(predmeat$xpos, levels = sort$xpos, labels = sort$adis)
predmeat$Rating <- factor(predmeat$Rating, levels = 0:3)
mypalette <- rev(c(rev(brewer.pal(3, "Reds")), "transparent"))
# Stacked bar chart using ggplot2:
ahp.meat <- ggplot(data = predmeat, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on meat") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); ahp.meat
#-------------------------------------------------------------------------------------------------------
library(gridExtra)
```

```
ahp.milkmeat <- windows(); grid.arrange(fpr.milk, ahp.milk, fpr.meat, ahp.meat, ncol = 2,
nrow = 2)
#-------------------------------------------------------------------------------------------------------------
# Cost probability calculation
fpr.cost <- clm(cost ~ adis, data = fpra)
# Model-based probabilities:
ecost <- emmeans(fpr.cost, ~ adis | cut, mode = "linear.predictor")
predcost <- rating.emmeans(ecost, type = "prob")
head(predcost)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predcost[predcost$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predcost with xpos:
predcost$xpos <- sort$xpos[match(predcost$adis, sort$adis)]
predcost$Rating <- factor(predcost$Rating, levels = 0:8)
predcost$xpos <- factor(predcost$xpos, levels = sort$xpos, labels = sort$adis)
mypalette <- rev(c(rev(brewer.pal(8, "Greens")), "transparent"))
# Stacked bar chart using ggplot2:
fpr.cost <- ggplot(data = predcost, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on cost") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); fpr.cost
#-------------------------------------------------------------------------------------------------------------
ahp.cost <- clm(cost ~ adis, data = ahpr)

# Model-based probabilities:
emcost <- emmeans(ahp.cost, ~ adis | cut, mode = "linear.predictor")
predcost <- rating.emmeans(emcost, type = "prob")
head(predcost)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predcost[predcost$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
```

```
# Update data frame predcost with xpos:
predcost$xpos <- sort$xpos[match(predcost$adis, sort$adis)]
predcost$xpos <- factor(predcost$xpos, levels = sort$xpos, labels = sort$adis)
predcost$Rating <- factor(predcost$Rating, levels = 0:6)
mypalette <- rev(c(rev(brewer.pal(6, "Reds")), "transparent"))
# Stacked bar chart using ggplot2:
ahp.cost <- ggplot(data = predcost, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on cost") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); ahp.cost
#-------------------------------------------------------------------------------------------------------
# Morbidity probability calculation

fpr.morbidity <- clm(morbidity ~ adis, data = fpra)
# Model-based probabilities:
emorbidity <- emmeans(fpr.morbidity, ~ adis | cut, mode = "linear.predictor")
predmorbidity <- rating.emmeans(emorbidity, type = "prob")
head(predmorbidity)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmorbidity[predmorbidity$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmorbidity with xpos:
predmorbidity$xpos <- sort$xpos[match(predmorbidity$adis, sort$adis)]
predmorbidity$Rating <- factor(predmorbidity$Rating, levels = 0:8)
predmorbidity$xpos <- factor(predmorbidity$xpos, levels = sort$xpos, labels = sort$adis)
mypalette <- rev(c(rev(brewer.pal(8, "Greens")), "transparent"))
# Stacked bar chart using ggplot2:
fpr.morbidity <- ggplot(data = predmorbidity, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on morbidity") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
```

```
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); fpr.morbidity
#-------------------------------------------------------------------------------------------------------
ahp.morbidity <- clm(morbidity ~ adis, data = ahpr)
# Model-based probabilities:
emorbidity <- emmeans(ahp.morbidity, ~ adis | cut, mode = "linear.predictor")
predmorbidity <- rating.emmeans(emorbidity, type = "prob")
head(predmorbidity)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmorbidity[predmorbidity$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmilk with xpos:
predmorbidity$xpos <- sort$xpos[match(predmorbidity$adis, sort$adis)]
predmorbidity$xpos <- factor(predmorbidity$xpos, levels = sort$xpos, labels = sort$adis)
predmorbidity$Rating <- factor(predmorbidity$Rating, levels = 0:6)
mypalette <- rev(c(rev(brewer.pal(6, "Reds")), "transparent"))
# Stacked bar chart using ggplot2:
ahp.morbidity <- ggplot(data = predmorbidity, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on morbidity") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); ahp.morbidity
#-------------------------------------------------------------------------------------------------------
library(gridExtra)

ahp.costmorb <- windows(); grid.arrange(fpr.cost, ahp.cost, fpr.morbidity, ahp.morbidity,
ncol = 2, nrow = 2)
#-------------------------------------------------------------------------------------------------------
# Mortality probability calculation
fpr.mortality <- clm(mortality ~ adis, data = fpra)
# Model-based probabilities:
emortality <- emmeans(fpr.mortality, ~ adis | cut, mode = "linear.predictor")
predmortality <- rating.emmeans(emortality, type = "prob")
```

```
head(predmortality)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmortality[predmortality$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmortality with xpos:
predmortality$xpos <- sort$xpos[match(predmortality$adis, sort$adis)]
predmortality$Rating <- factor(predmortality$Rating, levels = 0:10)
predmortality$xpos <- factor(predmortality$xpos, levels = sort$xpos, labels = sort$adis)
mypalette <- rev(c(rev(brewer.pal(10, "Greens")), "transparent"))
# Stacked bar chart using ggplot2:
fpr.mortality <- ggplot(data = predmortality, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
  ylab("Probability of impact on mortality") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")
windows(); fpr.mortality
#-------------------------------------------------------------------------------------------------------------
ahp.mortality <- clm(mortality ~ adis, data = ahpr)
# Model-based probabilities:
emortality <- emmeans(ahp.mortality, ~ adis | cut, mode = "linear.predictor")
predmortality <- rating.emmeans(emortality, type = "prob")
head(predmortality)
# We want the order of diseases (listed on the horizontal axis of the stacked bar chart) to be
sorted in order of Rating = 0:
sort <- predmortality[predmortality$Rating == 0,]
sort <- sort[sort.list(sort$Prob, decreasing = FALSE),]
sort$xpos <- 1:nrow(sort)
sort
# Update data frame predmortality with xpos:
predmortality$xpos <- sort$xpos[match(predmortality$adis, sort$adis)]
predmortality$xpos <- factor(predmortality$xpos, levels = sort$xpos, labels = sort$adis)
predmortality$Rating <- factor(predmortality$Rating, levels = 0:10)
mypalette <- rev(c(rev(brewer.pal(10, "Reds")), "transparent"))
# Stacked bar chart using ggplot2:
ahp.mortality <- ggplot(data = predmortality, aes(x = xpos, y = Prob, fill = Rating)) +
  geom_bar(stat = "identity", col = "grey") +
  xlab("Health issue") +
```

```
  ylab("Probability of impact on mortality") +
  scale_fill_manual(values = mypalette) +
  theme(axis.text.x = element_text(size = 10, angle = 90), axis.title.x = element_text(size =
12)) +
  theme(axis.text.y = element_text(size = 10, angle = 0), axis.title.y = element_text(size =
12)) +
  theme(legend.text = element_text(size = 10), legend.title = element_text(size = 12)) +
  labs(fill = "Score")

windows(); ahp.mortality
#================================
```

Data S2.2

prano	dist	vill	dis	pwise	milk	meat	cost	mort	morb
1	OKARA	22-GD	FMD	5	4	1	4	0	1
1	OKARA	22-GD	Haemorrhagic septicaemia	4	0	0	3	7	0
1	OKARA	22-GD	Mastitis	1	4	0	5	0	1
1	OKARA	22-GD	Red water	2	1	1	4	4	0
1	OKARA	22-GD	Ticks	3	4	2	3	0	1
2	OKARA	50/3R	FMD	5	3	3	0	0	4
2	OKARA	50/3R	Haemorrhagic septicaemia	4	5	0	0	5	0
2	OKARA	50/3R	Mastitis	2	6	0	4	0	0
2	OKARA	50/3R	Red water	1	0	0	0	10	0
2	OKARA	50/3R	Ticks	3	2	2	4	0	2
3	OKARA	40/3R	FMD	2	3	2	2	0	3
3	OKARA	40/3R	Haemorrhagic septicaemia	4	0	0	0	10	0
3	OKARA	40/3R	Mastitis	1	6	0	4	0	0
3	OKARA	40/3R	Red water	3	2	6	0	2	0
3	OKARA	40/3R	Ticks	5	2	2	0	0	6
4	OKARA	45-GD	Haemorrhagic septicaemia	2	0	0	0	10	0
4	OKARA	45-GD	Mastitis	5	5	0	3	0	2
4	OKARA	45-GD	Red water	3	2	3	3	2	0
4	OKARA	45-GD	Reproductive disorders	4	2	0	3	3	2
4	OKARA	45-GD	Ticks	1	4	3	3	0	0
5	OKARA	43-GD	FMD	4	5	2	2	0	1
5	OKARA	43-GD	Haemorrhagic septicaemia	3	0	0	0	10	0
5	OKARA	43-GD	Mastitis	1	5	0	5	0	0
5	OKARA	43-GD	Reproductive disorders	2	5	0	3	0	2
5	OKARA	43-GD	Ticks	5	2	2	2	0	4
6	JHELUM	Toba	Black quarter	5	0	4	3	3	0
6	JHELUM	Toba	FMD	3	2	2	2	2	2
6	JHELUM	Toba	Haemorrhagic septicaemia	4	0	0	0	10	0
6	JHELUM	Toba	Mastitis	1	5	2	3	0	0
6	JHELUM	Toba	Ticks	2	3	3	2	0	2
7	JHELUM	Noonan Wala	FMD	4	4	0	4	0	2
7	JHELUM	Noonan Wala	Haemorrhagic septicaemia	5	0	0	0	10	0
7	JHELUM	Noonan Wala	Internal parasitism	3	2	4	0	1	3
7	JHELUM	Noonan Wala	Mastitis	2	5	0	3	0	2
7	JHELUM	Noonan Wala	Reproductive disorders	1	0	0	8	0	2
8	JHELUM	Kotli Peeran	FMD	4	3	3	0	0	4
8	JHELUM	Kotli Peeran	Internal parasitism	2	0	7	0	0	3
8	JHELUM	Kotli Peeran	Mastitis	1	5	0	2	0	3
8	JHELUM	Kotli Peeran	Reproductive disorders	5	2	0	5	0	3
8	JHELUM	Kotli Peeran	Ticks	3	3	3	2	0	2
9	JHELUM	Daulat Pur	Black quarter	2	0	3	2	5	0

9	JHELUM	Daulat Pur	FMD	5	3	3	2	0	2
9	JHELUM	Daulat Pur	Mastitis	1	5	0	2	0	3
9	JHELUM	Daulat Pur	Red water	3	3	1	2	3	1
9	JHELUM	Daulat Pur	Reproductive disorders	4	4	1	1	2	2
10	JHELUM	Chundh	Black quarter	2	3	3	0	4	0
10	JHELUM	Chundh	FMD	3	3	3	1	1	2
10	JHELUM	Chundh	Mastitis	4	4	0	4	0	2
10	JHELUM	Chundh	Reproductive disorders	5	4	0	1	2	3
10	JHELUM	Chundh	Ticks	1	3	3	0	0	4
11	Bahawalpur	86-F	Black quarter	5	0	0	0	10	0
11	Bahawalpur	86-F	FMD	4	2	3	3	1	1
11	Bahawalpur	86-F	Mastitis	3	5	0	3	0	2
11	Bahawalpur	86-F	Reproductive disorders	1	6	0	2	0	2
11	Bahawalpur	86-F	Ticks	2	2	3	2	1	2
12	Bahawalpur	57-F	Black quarter	5	4	4	0	0	2
12	Bahawalpur	57-F	FMD	4	3	2	1	1	2
12	Bahawalpur	57-F	Mastitis	1	4	0	3	0	3
12	Bahawalpur	57-F	Reproductive disorders	2	4	0	1	2	3
12	Bahawalpur	57-F	Ticks	3	2	2	2	2	2
13	Bahawalpur	64-F	FMD	3	3	1	0	1	5
13	Bahawalpur	64-F	Haemorrhagic septicaemia	4	0	0	2	6	2
13	Bahawalpur	64-F	Internal parasitism	2	2	3	2	1	2
13	Bahawalpur	64-F	Mastitis	1	4	0	3	0	3
13	Bahawalpur	64-F	Red water	5	2	2	2	3	1
14	Bahawalpur	Basti Fareedi	Black quarter	1	2	2	2	2	2
14	Bahawalpur	Basti Fareedi	FMD	4	3	1	2	1	3
14	Bahawalpur	Basti Fareedi	Impaction	2	0	0	0	10	0
14	Bahawalpur	Basti Fareedi	Mastitis	3	5	0	3	0	2
14	Bahawalpur	Basti Fareedi	Ticks	5	3	2	2	1	2
15	Bahawalpur	Basti Nawan Khooh	Black quarter	1	1	0	0	7	2
15	Bahawalpur	Basti Nawan Khooh	FMD	4	3	0	1	1	5
15	Bahawalpur	Basti Nawan Khooh	Haemorrhagic septicaemia	2	0	0	0	10	0
15	Bahawalpur	Basti Nawan Khooh	Reproductive disorders	5	2	0	5	0	3
15	Bahawalpur	Basti Nawan Khooh	Ticks	3	2	3	2	0	3
16	Layyah	Basti Khairy Wala	Black quarter	5	0	4	0	6	0
16	Layyah	Basti Khairy Wala	FMD	3	2	3	1	0	4
16	Layyah	Basti Khairy Wala	Red water	4	2	2	2	2	2
16	Layyah	Basti Khairy Wala	Reproductive disorders	1	2	0	6	0	2
16	Layyah	Basti Khairy Wala	Ticks	2	3	3	0	0	4
17	Layyah	Basti Kalian	Black quarter	1	0	0	0	8	2
17	Layyah	Basti Kalian	FMD	2	4	1	0	1	4
17	Layyah	Basti Kalian	Red water	4	2	1	2	4	1
17	Layyah	Basti Kalian	Reproductive disorders	3	2	0	0	1	7
17	Layyah	Basti Kalian	Ticks	5	3	2	1	0	4
18	Layyah	82-ML	Black quarter	4	0	0	0	10	0

18	Layyah	82-ML	FMD	1	4	0	1	2	3
18	Layyah	82-ML	Red water	3	4	1	3	2	0
18	Layyah	82-ML	Ticks	5	2	2	1	0	5
18	Layyah	82-ML	Warble fly	2	4	2	1	0	3
19	Layyah	248-TDA	Black quarter	1	0	3	0	7	0
19	Layyah	248-TDA	FMD	2	6	0	1	0	3
19	Layyah	248-TDA	Red water	3	2	0	4	4	0
19	Layyah	248-TDA	Reproductive disorders	4	0	0	6	0	4
19	Layyah	248-TDA	Ticks	5	0	4	1	0	5
20	Layyah	Basti Balochan wali	Black quarter	1	0	3	0	5	2
20	Layyah	Basti Balochan wali	FMD	2	3	0	0	1	6
20	Layyah	Basti Balochan wali	Mastitis	3	4	1	3	0	2
20	Layyah	Basti Balochan wali	Red water	5	2	2	3	2	1
20	Layyah	Basti Balochan wali	Reproductive disorders	4	3	0	6	0	1
21	Thatta	Siddique Narejo	FMD	4	3	2	2	0	3
21	Thatta	Siddique Narejo	Haemorrhagic septicaemia	5	0	0	2	8	0
21	Thatta	Siddique Narejo	Internal parasitism	1	2	2	2	0	4
21	Thatta	Siddique Narejo	Mastitis	2	5	0	3	0	2
21	Thatta	Siddique Narejo	Ticks	3	2	4	1	0	3
22	Thatta	Haji Ahmed Soomro	FMD	4	3	1	0	2	4
22	Thatta	Haji Ahmed Soomro	Haemorrhagic septicaemia	3	0	0	2	8	0
22	Thatta	Haji Ahmed Soomro	Internal parasitism	2	2	3	0	0	5
22	Thatta	Haji Ahmed Soomro	Mastitis	1	4	0	3	0	3
22	Thatta	Haji Ahmed Soomro	Ticks	5	2	1	3	0	4
23	Thatta	Khalifa Ahmed	FMD	5	3	2	2	1	2
23	Thatta	Khalifa Ahmed	Haemorrhagic septicaemia	4	0	0	3	4	3
23	Thatta	Khalifa Ahmed	Internal parasitism	1	3	3	1	0	3
23	Thatta	Khalifa Ahmed	Mastitis	3	4	0	3	0	3
23	Thatta	Khalifa Ahmed	Reproductive disorders	2	0	0	5	0	5
24	Thatta	Hamzo Khaskhaily	FMD	3	2	1	1	2	4
24	Thatta	Hamzo Khaskhaily	Haemorrhagic septicaemia	4	0	0	2	6	2
24	Thatta	Hamzo Khaskhaily	Internal parasitism	1	3	2	3	0	2
24	Thatta	Hamzo Khaskhaily	Mastitis	5	4	0	3	0	3
24	Thatta	Hamzo Khaskhaily	Pneumonia	2	2	2	2	2	2
25	Thatta	Ahmed Khan Hathyar	FMD	1	2	2	2	1	3
25	Thatta	Ahmed Khan Hathyar	Haemorrhagic septicaemia	5	0	0	2	6	2
25	Thatta	Ahmed Khan Hathyar	Internal parasitism	2	3	3	1	1	2
25	Thatta	Ahmed Khan Hathyar	Mastitis	4	6	0	2	0	2
25	Thatta	Ahmed Khan Hathyar	Red water	3	0	6	1	0	3
26	Sukkur	Bagarji	Anthrax	3	0	0	0	6	4
26	Sukkur	Bagarji	FMD	2	3	2	2	1	2
26	Sukkur	Bagarji	Haemorrhagic septicaemia	4	0	1	2	4	3
26	Sukkur	Bagarji	Internal parasitism	1	3	3	2	1	1
26	Sukkur	Bagarji	Mastitis	5	6	0	4	0	0
27	Sukkur	Shah Kurripur	FMD	4	3	3	1	0	3

27	Sukkur	Shah Kurripur	Haemorrhagic septicaemia	2	0	0	0	7	3
27	Sukkur	Shah Kurripur	Internal parasitism	1	3	2	2	0	3
27	Sukkur	Shah Kurripur	Mastitis	3	4	0	3	0	3
27	Sukkur	Shah Kurripur	Ticks	5	1	1	0	0	8
28	Sukkur	Haji Nazar M. Soomro	FMD	4	3	2	2	1	2
28	Sukkur	Haji Nazar M. Soomro	Haemorrhagic septicaemia	3	0	0	1	6	3
28	Sukkur	Haji Nazar M. Soomro	Internal parasitism	2	2	3	2	1	2
28	Sukkur	Haji Nazar M. Soomro	Mastitis	5	5	1	3	0	1
28	Sukkur	Haji Nazar M. Soomro	Reproductive disorders	1	0	0	6	0	4
29	Sukkur	Dakhano Phulphoto	Black quarter	5	3	4	2	0	1
29	Sukkur	Dakhano Phulphoto	FMD	4	2	2	1	0	5
29	Sukkur	Dakhano Phulphoto	Haemorrhagic septicaemia	3	0	0	1	5	4
29	Sukkur	Dakhano Phulphoto	Internal parasitism	2	3	2	2	1	2
29	Sukkur	Dakhano Phulphoto	Ticks	1	3	3	1	0	3
30	Sukkur	Gharro goth	FMD	5	4	1	1	1	3
30	Sukkur	Gharro goth	Haemorrhagic septicaemia	1	0	0	0	7	3
30	Sukkur	Gharro goth	Internal parasitism	2	3	1	3	1	2
30	Sukkur	Gharro goth	Reproductive disorders	3	2	0	5	0	3
30	Sukkur	Gharro goth	Ticks	4	1	2	2	1	4

Data S2.3

mno	dist	ppt	dis	pwise	milk	meat	cost	mort	morb
1	OKARA	VO	Ticks	1	2	2	2	0	4
1	OKARA	VO	Blood parasites	2	1	3	2	2	2
1	OKARA	VO	FMD	3	4	0	3	0	3
1	OKARA	VO	Reproductive disorders	4	4	0	4	0	2
1	OKARA	VO	Mastitis	5	4	0	4	0	2
2	OKARA	VA	Mastitis	1	5	0	5	0	0
2	OKARA	VA	Internal parasitism	2	3	2	3	0	2
2	OKARA	VA	FMD	3	2	2	2	2	2
2	OKARA	VA	Blood parasites	4	2	2	3	1	2
2	OKARA	VA	Red water	5	3	2	3	0	2
3	JHELUM	VO	Reproductive disorders	1	2	0	6	0	2
3	JHELUM	VO	Mastitis	2	4	1	3	0	2
3	JHELUM	VO	Ticks	3	2	2	1	1	4
3	JHELUM	VO	FMD	4	3	1	1	1	4
3	JHELUM	VO	Internal parasitism	5	1	3	2	1	3
4	JHELUM	VA	Ticks	1	4	0	3	0	3
4	JHELUM	VA	Mastitis	2	6	0	2	0	2
4	JHELUM	VA	FMD	3	5	1	1	0	3
4	JHELUM	VA	Warble fly	4	1	2	1	0	6
4	JHELUM	VA	Black quarter	5	1	2	0	7	0
5	BAHAWALPUR	VO	Reproductive disorders	1	0	0	6	0	4
5	BAHAWALPUR	VO	Mastitis	2	5	1	3	0	2
5	BAHAWALPUR	VO	Internal parasitism	3	2	3	0	0	5
5	BAHAWALPUR	VO	Ticks	4	2	2	2	0	4
5	BAHAWALPUR	VO	Tympany	5	4	0	1	2	3
6	BAHAWALPUR	VA	Reproductive disorders	1	2	0	6	0	2
6	BAHAWALPUR	VA	Ticks	2	3	2	2	0	3
6	BAHAWALPUR	VA	Arthritis	3	2	0	5	0	3
6	BAHAWALPUR	VA	Mastitis	4	3	0	5	0	2
6	BAHAWALPUR	VA	Red water	5	2	1	2	3	2
7	LAYYAH	VO	Mastitis	1	4	0	4	0	2
7	LAYYAH	VO	FMD	2	4	0	0	1	5
7	LAYYAH	VO	Blood parasites	3	2	2	2	3	1
7	LAYYAH	VO	Black quarter	4	0	3	1	4	2
7	LAYYAH	VO	Milk Fever	5	4	0	2	1	3
8	LAYYAH	VA	Mastitis	1	5	0	3	0	2
8	LAYYAH	VA	Blood parasites	2	4	1	3	2	0
8	LAYYAH	VA	Black quarter	3	0	2	0	8	0
8	LAYYAH	VA	FMD	4	2	1	1	3	3
8	LAYYAH	VA	Haemorrhagic septicaemia	5	0	0	0	10	0
9	THATTA	VO	Internal parasitism	1	6		0	2	2

9	THATTA	VO	FMD	2	6	0	1	1	2
9	THATTA	VO	Mastitis	3	5	0	4	0	1
9	THATTA	VO	Blood parasites	4	1	2	2	3	2
9	THATTA	VO	Haemorrhagic septicaemia	5	0	0	2	7	1
10	THATTA	VA	Internal parasitism	1	1	0	1	2	6
10	THATTA	VA	Mastitis	2	4	1	4	0	1
10	THATTA	VA	FMD	3	4	2	0	1	3
10	THATTA	VA	Haemorrhagic septicaemia	4	2	0	3	5	0
10	THATTA	VA	Red water	5	2	2	3	3	0
11	SUKKUR	VO	Internal parasitism	1	3	2	1	0	4
11	SUKKUR	VO	FMD	2	2	1	1	0	6
11	SUKKUR	VO	Reproductive disorders	3	1	1	3	1	4
11	SUKKUR	VO	Mastitis	4	7	0	2	0	1
11	SUKKUR	VO	Haemorrhagic septicaemia	5	0	0	2	6	2
12	SUKKUR	VA	Internal parasitism	1	3	2	1	0	4
12	SUKKUR	VA	FMD	2	3	3	0	0	4
12	SUKKUR	VA	Mastitis	3	4	1	3	0	2
12	SUKKUR	VA	Haemorrhagic septicaemia	4	3	0	0	6	1
12	SUKKUR	VA	Anthrax	5	1	0	0	8	1