IMMUNOPROPHYLAXIS OF TICK INFESTATION IN BOVINE

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I dedicate

this effort to

my late daughter , Isha and my parents.

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LIST OF ABBREVIATIONS

AGPT	**Agar Gel Precipitation Test**
BLAST	**Basic Local Alignment Search Tool**
BSA	**Bovine Serum Albumin**
CFT	**Complement Fixation Test**
EDTA	**Ethylene Diamine Tetraacetic Acid**
ELISA	**Enzyme Linked Immuno Sorbant Assay**
FAO	**Food and Agricultural Organization**
GM	**Gut Membrane**
GMT	**Geo Metric Mean**
IPM	**Integrated Pest/Vector Management**
ITE	**Intestinal Tissue Extract**
IVM	**Ivermectin**
kDa	**Kilo Dalton**
KPK	**Khyber Pakhtoon Khwa**
MPSP	**Major Piroplasm Surface Protein**
MSP	**Major Surface Protein**
OB	**Oil Based**
OPD	**'O' Phenylene Diamine**
PCR	**Polymerase Chain Reaction**
PCV	**Packed Cell Volume**
RBC'S	**Red Blood Cells**
SDS	**Sodium Dodycyle Sulphate**
SGE	**Salivary Gland Extract**
TBD'S	**Tick Borne Diseases**
TOSP-AGP	**Tissue Organ Specific Agar Gel Precipitin**
TTO	**Tea Tree Oil**
WHTE	**Whole Homogenate Tissue Extract**
WTHS	**Whole Tick Homogenate Suspension**

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CHPTER 1

INTRODUCTION

Tick infestation in livestock is a common problem in countries having hot and humid environment. Exotic cattle breeds are more vulnerable to tick infestation as compared to local stocks due to non-adaptive immune factors, grooming activity, skin color and thickness, area of skin available for infestation or length of fur (Mattioli, 1998 and Meltzer, 1996). Tick infestation not only reduces appetite and body condition of the host but also reduces the hide quality up to 20-30% in leather industry (Ghosh *et al.,* 2007). Even ticks with smaller mouthparts like *Rhipicephalus (Boophilus)* may cause similar losses when present in large number. Ticks with long and massive mouthparts such as *Amblyomma* and *Hyalomma* may induce abscesses, lameness and loss of teats in their hosts. The saliva of certain ticks like *Dermacenter* can cause toxicosis and paralysis as their salivary secretions contain toxins (Jongejan and Uilenberg, 2004).

Tick infestation also causes severe anemia, loss of production, weakness and immuno-suppression in cattle and buffaloes (Gwakisa *et al.,* 2001). Moreover, it can cause transmission of viral, bacterial and protozoan pathogens causing diseases like hemorrhagic fever, ehrlichiosis, anaplasmosis, theileriosis and babesiosis in meat and dairy animals. It has been studied that about 80% of the world cattle population is infested with ticks (Bowman *et al.,* 1996) and Food and Agricultural Organization (FAO)of the United Nation estimated the global cost of hard tick infestation to be US $ 7.0 billion annually (Harrow *et al.,* 1991). Proposed new agents producing

clinical signs are increasingly reported from ticks mainly by sequencing Poly Chain Reaction products and doing comparison of these sequences by BLAST in GeneBank, (Telfor III and Goethert, 2004).

Ticks belong to the order Acarina and class Arachnida. Two families of ticks exist, the hard ticks (Ixodidae) with 670 species and soft ticks (Argicidae) about 150 species (Urquhart et al., 1996). In Pakistan various species of ticks of the genera *Rhipecephalus, Boophilus, Hyalomma, Amblyomma* and *Hemaphysalis* have been reported to infest the dairy and meat animals. Breed, age, sex, environmental conditions, nutritional and lactation status of an animal, however, are the factors that mainly determine the density of tick infestation (Sajid *et al.,* 2008). The prevalence of ticks has been found higher in May through August which is the hottest duration of the summer in Pakistan. The most favorable sites for tick attachment are udder, ear, neck, inside of thighs and vulvolar regions (Yakhachali *et al.,* 2004)

Injectables, burning of pastures, selection of breeds with a higher natural resistance to tick infestation and applications of acaricides are different methods for tick control typically used in Pakistan. Application of acaricides is the most common way to control tick infestation which is applied in the form of a spray, shower or spot on. This method is not sustainable due to expensiveness, residual effect and development of resistance against many tick species (Makala *et al.,* 2003). The resistance to ticks is primarily an acquired rather than innate (Wagland, 1978). Moreover acaricides cause environmental pollution and health hazards to persons applying acaricides.

An important component of integrated pest/vector management (IPM) is immunoprophylaxis (Ghoshe *et al.,* 2007). Different types of vaccines have been developed throughout the world to control tick infestation in cattle and buffaloes. They include whole tick lysate vaccine, tick cement protein vaccine, and recombinant tick vaccine (Bishop *et al.,* 2002). Progress in developing suitable anti tick vaccines is slow primarily due to difficulty in identifying suitable sources of antigens. Concealed as well as exposed antigens have been used to develop vaccine against different tick species. Attachment of ticks to a vaccinated host results into mitigation of engorgement weight and reproductive performance due to antibody binding with gut wall which causes its lysis (Nuttal *et al.,* 2004). Complete and well organized immunization regime may promote success in controlling tick burden to dairy and meat animals.

Pertinent literature regarding immunoprophylaxis of tick infestation is scanty. This study had therefore been designated with the following objectives.

1. Characterization of hard ticks collected from the area of study by observing its morphological features under microscope.
2. Development and evaluation of a vaccine prepared from field isolates of hard ticks.
3. Hard ticks collected from infected animals may be contaminated with blood borne parasites that might ultimately contaminate the tick vaccine. In this study, efforts therefore were made to identify the presence of *Theileria annulata* using a PCR technique in homogenized material of ticks. This parasite was chosen for the analysis

because it is the major parasite found in ticks and also has a significant impact on cross- breed animals in Pakistan.

4. Efforts were also made to grow intestinal epithelial cells of hard ticks as a monolayer for subsequent production of a vaccine.

CHAPTER 2

REVIEW OF LITERATURE

1. Tick Infestation

1.1 Tick classification, structure and bionomics

Most of vertebrates living on earth are susceptible to tick infestation; their warmth and odor are attractive to ticks (Harwood and James, 1979). Suborder Ixodidea and subclass aracri (Arachnida) contain two tick families *Ixodidae* and *Argasidae* (Urquhart, 1996; Soulsby, 2006 and Porto Neto *et al.,* 2011). *Ixodidae* can be differentiated from *Argasidae* by having a hard, chintinous shield or scutum. Scutum covers only the anterior part behind the capitulum in immature and female Ixodes, while in mature male it covers the dorsum fully. All tick species pass through four stages (egg, larva, nymph and adult) from six weeks to three years. The larva hatched from egg is six-legged and remain in this condition until moults into nymph. The nymph has four pair of legs and transforms into sexually matured adult. (Harwood and James, 1979; Urquhart, 1996 and Soulsby, 2006). Female ticks can become greatly distended and when fully engorged are bean shaped; they are 200 times greater in weight as compared to unengorged (Harwood and James, 1979 and Urquhart, 1996).

Ixodes tick species that remain on the host during the two molting periods are known as one-host ticks. In two host species, the molt to the nymphal stage occurs on the host but engorged nymph

leaves the host, molts in the environment and then finds a new host. In the three host tick life cycle, both the larvae and nymph leave the host to molt, attaching to host again after each molt (Urquhart, 1996 ; Minjauw and McLeod, 2003and Zajac *et al.,* 2006), while ticks of *Argasidae* are free living (Jongejan and Uilenberg, 2004).). Ixodes ticks lay eggs in batches, each batch contains upto 18000 eggs and female dies after hatching (Harwood and James, 1979 and Soulsby, 2006). In transtadial transmisstion, tick can transmit disease pathogens from one molt to another while in transovarion transmission, adult female can transmit pathogens to larva through infecation of ovaries (Bowman *et al.,* 2003). There are almost 900 species of ticks that are endemic to most continents (Barker and Murrell, 2004) and important are *Hyalomma anatolicum anatolicum, Boophilus (Rhipecephalus) microplus* and *Amblyomma americanum* (Porto Neto *et al.,* 2011 and *Atif et al.,* 2012). The highest mean pre-oviposition period was during spring while it was the lowest in autumn; the mean oviposition period was also the highest in spring. The incubation period of the ova of *Hyalomma species* varied in different seasons, no oviposition was recorded at the temperature 100ºC and 85% humidity. The maximum number of eggs was laid at 34ºC and the lowest egg production occurs at 15ºC. The maximum number of eggs hatches at 32ºC and 85% humidity. The variation in relative humidity has no appreciable effect on rate of development of ticks while the number of eggs laid increase with the rise in temperature (Durrani and Shakoori, 2009).

1.2 Prevalence study

1.2.1 Ambient environmental conditions for ticks

The ability of Ixodes ticks to survive off the host is astounding and much of this time is spent in an active state resembling to diapauses. Phenomenon of diapause is mostly found in temperate

countries, it may also occur in tropical countries. The prevalence to ticks is more in areas with higher humidity and main stress factor for their life is desiccation during off host period (Marquardt *et al.,* 2005). The highest tick infestation was recorded when mean temperature was 27^{o}C and relative humidity as 85% in Rawalpind and Islamabad areas of Pakistan during month of June-July (Rehman *et al.,* 2004).

1.2.2 Prevalence study in Pakistan

Tick infestation study was conducted in cattle, buffaloes, goats and sheep in Rawalpindi and Islamabad regions. It showed prevalence of five species namely, *Haemaphysalis (H) sulcata, H. anatolicum, H. anatolicum anatolicum, H. marginatum* and *Haemaphysalis (R) erinacei* at 74%, 14%, 12%, 1% and at1%, respectively. They reported the prevalence of *Haemaphysalis (H) sulcata and H. anatolicum* highly significant (Rehman *et al.,* 2004).In Peshawar district of Khyber Pakhtoon Khwa (KPK) province the hard tick prevalence was conducted in randomly selected livestock farms. It was found that overall 13% of the observed farm animals were tick infested showing the highest infestation in cattle (20%) followed by sheep (13%), goats (12%), buffaloes (11%) and donkeys (six %). The highly prevalent ticks were *Boophilus species* (46%) followed by *Hyalomma species* (31%), *Rhipicephalus species* (18%) and *Amblyomma species* (five %). It was found that tick infestation was higher in late summer and lower in winter (Manan *et al.* 2007). Similar study was conducted in Layyah and Muzaffargarh districts in population of cattle, buffaloes, sheep, and goats. The cattle at 75% were found the highest followed by goats at 52% and buffaloes at 40%, respectively. No case of tick infestation was found in camels and sheep. *Hyalomma anatolicum* was the species which was the most abundant followed by

Rhipicephalus sanguineus (Sajid *et al.,* 2008). A survey to study the prevalence of *Hyalomma species* was conducted in Rawalpindi, Multan and Lahore districts of Punjab province in Pakistan. In cattle genera wise study showed the highest prevalence (12%) of *Hyalomma species* and the lowest prevalence (four %) of *Rhipicephalus species.* The results showed the highest prevalence (67%) of ticks in district Lahore. A survey was conducted to study the prevalence of *Hyalomma species* in Rawalpindi, Multan and Lahore districts of Punjab province in Pakistan (Durrani and Shakoori, 2009). In Sargodha, Khushab and Rawalpindi districts of Punjab province, overall prevalence of cattle tick infestation was 54.76%. The prevalence of *Hyalomma anatolicum anatolicum, Rhipicephalus (boophilus) microplus, Rhipicephalus (boophilus) annulatus and Haemaphysalis species* were reported in this study (Atif *et al.,* 2012).

1.2.3 Prevalence study in other countries

In Oshnavich suburb, West Azerbaijan Ixodid tick distributions per animal were 5, 3-4, 4-5, 2-3 and 1-2 in cattle, calves, buffaloes, female buffaloes and sheep, respectively. The prevalence of ticks was 44%, 41% and 47% in cattle, buffaloes and sheep, respectively. Two genera of ixodid ticks were found in cattle, *Hyalomma* at (64%) and *Rhipicephalus* at (four %). Six species were found in cattle, *Hyalomma anatolicum excavatum* (four %), *H. anatolicum anatolicum* (five %), *H. asiaticum asiaticum* (16%), *H. marginatum* (four %), *H. dromedarii* (13%) and *Rhipicephalus bursa* (four %). *Hyalomma species* (63%) and *Rhipicephalus species* (four %) were also present in buffaloes. Five genera of ixodid ticks are found in sheep, *Hyalomma species* (two %), *R. species* (23%), *Haemaphysalis species* (two %), *Dermacentor species* (27%) and *Boophilus species*(two %) (Yakhachali *et al.,* 2004). In Mazandran provine, Iran nine species were

identified: *Rhipicephalus (Boophilus) microplus* (51.3%), *Rhipicephalus bursa* (16.8%), *Haemaphysalis punctata* (6.3%), *Ixodes ricinus* (68%), *Hyalomma anatolicum* (12.5%), *Hyalomma anatolicum exacavatum* (5.2%), *Hyalomma asiaticum* (0.6%), *Hyalomma detrium* (0.2%) and *Dermacenter species* (0.1%). *Boophilus (Rhipicephalus) annulata*, *Rhipicephalus bursa* and *Hyalomma species* were dominant in the study area (Razmi *et al.,* 2007). In different upazila of Chittagong District from a total number of 380 cattle were examined, of which 138 (36.31%) cattle were found infested. Three species of ticks were identified namely *Boophilus microplus*, *Rhipicephalus sanguineus* and *Haemaphysalis bispinosa*. . Tick infestation was more prevalent in local (43.82%) cattle than the cross-bred (24.13%) cattle (Kabir *et al.,* 2011).

1.3 Host susceptibility to tick infestation

Bos indicus are naturally more resistant to tick infestation as compared to *Bos Taurus*. Less than one % of ticks feed successfully on *Bos indicus* (local breed), while response of *Sahiwal* breed against control measures was significant. In case of *Bos taurus* breeds more than 50% may feed to repletion. Tick infestation effects differ considerable due to variation in susceptibility and resistance to the host, which are also influenced by grooming and grazing behavior, the difference in quality and quantity of grazing and the timing and abundance of tick burden (Latif and Pegram, 1992). Taurine crosses with zebu were more vulnerable to ticks as compared to pure zebu cattle under similar field conditions. This tick resistant in Zebu cattle may be due to presence of significantly higher serums complement level in their blood as compared to crossbred cattle (Wambura *et al.,* 1998). It was documented the highest tick incidence in young

calves at 48%, following growing stock at41%, heifer at 40% and adult cattle at 35% (Das, 1994).

1.4 Ticks as potential vectors of pathogens

1.4.1 Pathogens vector by ticks

The ecology and physiology of ticks have made them second most important vectors after mosquitoes. Ticks transmit a large variety of intercellular bacteria in the *Rickettsia* group like *Rickettsia, Ehrlichia* and *Anaplasma*. Similarly several piroplasm protozoa like *T. annulata, T.parva* and *Babesiosis bigemmina* are also transmitted specifically by ticks (Marquardat *et al.,* 2005). Tropical theileriosis is disease of cattle and caused by *Theileria annulata*; the disease is transmitted mainly by *H. detritum* and *Hyalomma excavatum* (Fesharki, 1988). Transmission of *Anaplasma marginale* occurs by ticks, both male tick and host become persistently infected with *A.marginale* and serve as reservoir of infection. As erythrocytes are major site of infection, *A. marginale* undergoes developmental cycle in the tick. The cycle begins in gut cells and transmission occurs to susceptible host through salivary glands (Kocan *et al.,* 2010).

1.4.2 Role of salivary gland in disease transmission

Ticks like other haematophagus arthropods must cope with coagulation, platelet aggregation and pain or itch responses for obtaining blood meal successfully (Ribeiero, 1995a). Salivary glands are necessary for the biological success of ticks during off-feed period and on-host period. Ticks return back to host about 70% of the fluid and ion components of blood meal during the phenomenon of salivation. Moreover, they also indicated that the salivary glands are sites of

pathogen development and ticks transmit different pathogens through saliva. The multi-functionary activities of salivary glands for tick survival and vector potential portray the gland as target for intervention (Bowman and Sauer, 2004). *Theileria* sporoblast was detected in salivary glands of *Hylomma detrium detrium.* Ninteen percent (24/127) of *Hylomma detrium detrium* were infected with *theileria species.* Among these infected ticks more than 50% had five or more sporoblasts in their salivary glands (Flach and Ouheli, 1992).

1.4.3 Detection of pathogens in ticks

PCR analysis was performed to detect of *Theileria annulata* in *Hyalomma* ticks using primers derived from a gene which code for 30kDa major merozoite surface antigen. It was found that infection rate in the adult ticks is high at 62% which was decreased to zero for one day in tick groups which were treated with Butalex ^{TM} and again increases to 30% two days later. Two hundred two adult ticks were collected from low theileria parasitemic zebu cattle from Mauritania and are tested for *Theileria annulata* by the PCR. In *H. dromedarii* 73% and 57% infection rates were detected from Gorgol and Trarza regions, respectively. While in Gorgol region seven percent *Hyalomma marginatum rufipes* are positive. These findings confirmed that PCR analysis was a useful tool for determining the infections in ticks and that *H. dromedarii* was the main vector of *T. annulata* in the area of study (D'Oliviera *et al.*, 1997). Twenty-eight field isolated theileria parasite DNAs were obtained from dairy and beef cattle in distinct geographical areas of Thailand and characterized them by using polymerase chain reaction amplification with six sets of oligonucleotide primers. Three sets of them are modified from two genes of immunodominant major piroplasm surface protein (MPSP) coding for 32kDa (p32) of *T. sergenti*

and 33/34kDa (p33/34) of *T. buffeli,* and MPSP of *Theileria* species (Thai-isolate). The other three sets of primers were basically generated from three alleles of MPSP which were specific for Japanese strains of theilerial organisms. The results indicated that 14 out of 28 isolates were amplified by the Thai-specific primers whereas six isolates were amplified by the p32 specific primers and the other five isolates were amplified by the p32 and Thai-specific primers. In addition, by using the allele-specific PCR, 14 out of 28 isolates contained C-type MPSP whereas three isolates contained B1 type parasites. Interestingly, 20 out of 28 isolates could be amplified by the Thai-specific primer. The majority of theileria parasites distributed in Thailand contained Thai type parasites, whereas C-type parasites showed the mixed population with B1 and Thai type parasites. No I-type parasite is detected (Sarataphan *et al*., 1999). *T. parva* infections in field-collected ticks and bovine samples from Tanzania were detected by PCR. They found that infection prevalence was high at 75% in unfed adult *Rhipicephalus appendiculatus*, but low at three percent in unfed adult *R. appendiculatus.* Tick infection prevalence rate was similar to that in previous studies in which *T. parva* was detected in salivary glands of field collected ticks by staining technique (Ogden *et al*., 2003). The potential of *R. appendiculatus* to transmit *T. parva* during different time periods was studied by using PCR analysis. In this experiment infected adult ticks were allowed to engorge to naive mice for graded lengths of time. They observed that infected ticks started to transmit *T. parva* after 72 hours of their attachment to mice and transmission of *T. parva* from ticks to mouse increased as duration of tick attachment increases. On day four, five, six and seven post-tick attachment, the transmission of the parasites was 78%, 100%, 85% and 100%, respectively (Konnai *et al.*,2007).

1.5 Tick Infestation Based Economic Losses

Over 250 million cattle population of world is under the threat of tropical theileriosis which is an important tick transmitted protozoan disease (Susana *et al.,* 1989). Acaricidal application for the control of ticks and tick-borne is costly and still leave treated animals susceptible to tick-borne diseases (Meltzer *et al.,* 1993). The maturity age in cows at first calving was 3.9 years in tick free herds while in tick infested herds it was 4.2 years. Similarly, the weight gain to adult tick free cow was 221kg in contrast to 215kg in tick infested adult cow. Mortality in cows in tick infested herds was five % while in tick free it was four %. Calving interval was 511days in tick free cows while in the tick infested cows it was 591 days (Pegram *et al.,* 1996). It was estimated that one fully engorged tick was responsible for the loss of 8.9ml in milk production and 1.0g of bodyweight per day during the trial period. In tick free cows, the dry matter intake was 0.83kg greater than that of infested cows. There was no significant difference in milk composition, packed cell volume (PCV) and total plasma protein (TPP) between control and experimental cows (Jonsson *et al.*, 1998). Eighty % of cattle population of world was at risk of tick infestation and estimate of productivity losses due to ticks and tick borne diseases was about US $ 7000 million (McCoskar, 2004). Estimated economic losses of tropical theileriosis in an endemic region of the North of Tunisia was about $9388.20; a major proportion of these economic losses (52%) were due to asymptomatic infection. The disease condition with clinical signs of anaemia led to highest losses in weight gain, whereas disease cases with typical signs led to economic losses of 24%; death was the most important element in this case (Gharbi *et al.,* 2006). Public health and economic importance of ticks had been established due to their

capability to transmit diseases to people and animals. In this way ticks cause great economic losses to livestock by adversely affecting livestock hosts in different ways. Anemia is direct effect of ticks as they act as potential vector for blood-protozoa and helminthes parasites. In heavily tick infested animals, there is a reduction in live weight and anemia among domestic animals, while their bites also reduce the quality of hides in leather industry. Major world-wide economic losses caused by ticks are due to their ability to transmit different viral, bacterial and protozoan pathogens (Iqbal *et al.*, 2006). Tick-borne diseases (TBDs) especially anaplasmosis, babesiosis, cowdriosis and theileriosis, constrain cattle production and improvement in Tanzania, leading to considerable economic losses due to production losses, treatment and control costs. The total annual national loss due to TBDs was estimated to be 364 million US $, including an estimated mortality of 1.3 million cattle. Theileriosis accounted for 68% of total loss, while anaplasmosis and babesiosis each accounted for 13% and cowdriosis accounted for six percent of the total loss. Cost associated with mortality, chemotherapy and acaricides application accounted for 49%, 2%, and 14% of the total estimated annual TBDs losses, respectively. Treatment cost, milk loss and weight loss accounted for one %, six % and nine % (Kivaria, 2006). Overall cost was as $4,096,000 per annum in 1998 to the dairy industry of Queensland due to cattle ticks and tick borne diseases. About 49% of this cost was incurred on control measures and 51% was due to losses in production (Jonsson *et al.* 2008).

2 Control of ticks

2 .1 Chemotherapy

Effects of four chemicals namely, coumaphos, diazinon, permethrin and ivermectin were evaluated on the basis of reduction in tick count on the body of host. It was proved that ivermectin was the best in efficacy at 83 to 86%, followed by diazinon at 67 to 70%, permethrin at 66 to 62% and coumaphos at 53 to 54%. These trials were performed in sheep and goats which were kept in experimental and control group (Khan *et al.,* 1998). The biocontrol agents play significant role towards limiting tick population. These agents include many bacteria, fungi, spiders, ants, beetles, rodents, birds and other living things. Fungi of the genera *Beauveria* and *Metarhizium* are the agents having good potential for controlling ticks (Samish and Rehacek, 1999). Ticks have numerous natural enemies, but only a few species have been evaluated as tick bio-control agents. The most promising entomopathogenic fungi appears to be *Metarhizium anisopole* and *Beauveria bassiana* strains which are already commercially available for the control of some pests (Samish *et al.*, 2004). Effect of essential oil of *Melaleucaalternifolia* (tea tree oil, TTO) at different doses and for different exposure times on nymphs of tick *Ixodes ricinus* was studied. A dose of eight micro liters TTO was found to be lethal for more than 70% of ticks when inhaled and this effect was increased when the dose was enhanced to 10µl (>80%). The findings of the experiments showed that TTO had acaricidal properties and could be potentially useful in controlling the ticks (Iori *et al.*, 2005). Efficacy of ivermectin and moxidectin by administering a single dose of each endectocide subcutaneously was determined. Ivermectin and moxidectin were administered at the dose rate of 200µg per kg live body weight to control all parasitic developmental stages of *Boophilus microplus* in cattle. The reproductive

capability of the surviving females was reduced by 99%, for each of endectocide. Treated females produced egg masses weighing six-times less than egg masses produced by female ticks in untreated group (Ronald *et al.*, 2005). Bioacaricidal activity of a recombinant baculovirus was tested which expressed a chitinase gene. The virus expresses a chitinase enzyme (AcMNPV-CHT1) from *Haemaphysalis longicornis* and found to have a novel acaricidal effects against ticks. Synergistically, recombinant virus was used with chitinase as a mixture. This mixture was found to kill ticks faster as compared to pure chitinase and recombinant virus independently. Moreover, it was also found that a mixture of recombinant virus and flumethrin could decrease the dose of the chemical acaricide to half (Assenga *et al.*, 2005). *Hyalomma anatolicum anatolicum* infestations in bovines was treated with ivermectin and cypermethrin and compared their efficacy of two drugs. After examining ticks, it was indicated that ivermectin and cypermethrin compounds were effective in vitro condition against *H. a. anatolicum*. On the other hands, cypermethrin pour-on showed a higher in vivo efficacy as compared to IVM after 15 days of treatment. During study it was observed that most of the farmers are using acaricides wrongfully and indiscriminately along with bad husbandry practices on their farms (Sajid *et al.*, 2009). The use of chemicals to control ticks usually results in induction of drug resistance, environment deterioration and increases cost treatment in addition to the necessity for laborious and repeated applications (de la Fuente *et al*., 1998). Field reports of development of ivermectin resistance in ticks (Martin *et al.*, 2001) also necessitate work on other biological tick control method.

2.2 Immunotherapy

2.2.1 Antigens

Immune response of membrane antigens extracted from the mid-gut (GM) of the *Boophilus microplus* was monitored in sheep and cattle by enzyme-linked immunosorbent assay (ELISA). Dose of one milligram of GM induces an antibody response in sheep comparable to that induced by five milligram GM. It was indicated single or divided doses of 5mg GM induced the same antibody levels in sheep during 12 weeks trial. Cattle vaccinated with either 500mg GM in two doses or with 50 or 500mg GM in three doses had detectable and significant antibody responses and protected as 89%, 80% and 95%, respectively against challenge tick infestation. In another experiment, cattle vaccinated with 2.95mg GM divided into 12 doses over six months had antibody levels that protected challenge infestation successfully at 96% (Jackson and Opdebeeck 1989). An experiment was performed to study cross protection of antigen Bm86, isolated from *B. microplus* against other *Boophilus species.* In many parts of the world, *B. microplus* existed with other *Boophilus species*, mainly *B. annulatus* and *B. decoloratus*. To test cross reactivity of Bm86 antigen vaccine against other *Boophilus species,* Gavac immunized cattle were challenged with *B. annulatus* larvae artificially. The results of the experiment proved high efficacy of Gavac in the control of *B. annulatus* infestations (Fragoso *et al.*, 1998). Possible interactions of the Bm86 with other vaccine antigens was studied. In an experiment a potent stimulatory effect of the recombinant Bm86 is demonstrated when Bm86 was administered with recombinant Hepatitis B surface antigen in mice and with inactivated infectious Bovine Rhinothraquetis virus in cattle. The results led to conclusion that Bm86 antigen was a good candidate for combining vaccines for cattle because of its immunogen and adjuvant role (Garcia *et al.*, 1998)**.** Tick saliva

of *Ixodes recinus* blocked hemolysis of sheep red blood cells by following alternative pathway of complement. Acquired immune response of BALB/c mice is also regulated by tick salivary pharmacopeia. Some constituents of salivary glands down regulated response of draining lymph node T cells and sensitized splenic T cells in vitro; the response of naive splenic T cells to Con A stimulation in vitro was also down regulated by tick saliva (Mejri *et al.,* 2001)**.** Glycoproteins of 34 kDa from the larvae of *H. anatolicum anatolicum* and 29 kDa were isolated from the larvae of *B. microplus*. In animals the supporting efficacy of the antigens with incomplete Freund's adjuvant gave protection against 56% of larvae and 52% of adults of *H. a. anatolicum*, respectively, whereas the efficacy was 40% against adults of *B. microplus*. Seventy percent and 64% efficacy was calculated against larvae and adults of *H. a. anatolicum*, respectively, when antigens were used in combinations. In case of adults of *B. microplus* this efficacy was 63%. Above 30 weeks protection period was indicated against *H. a. anatolicum* and *B. microplus* infestations when immunogens in combination with incomplete Freund's adjuvant were used (Ghosh *et al.,* 2005).

2.2.2 Development of immunity

In animals acquired immunity against tick infestation can be produced by two ways; repeated tick infestation and active immunization with crude or purified or recombinant antigens (Mulenga *et al.,* 2000). Tick resistance may lead to ten times reduction in engorgement weight of adult ticks and a significant decline in engorgement weight of the nymphs. *Rhipicephalus zambeziensis* resistant rabbits had no protection against *Amblyomma hebraeum*. Feeding of tick on resistant rabbits resulted into severe gut damage which was associated with the binding of host IgG to mid-gut cells and complement mediated lysis of such complexes. Abnormally fed

ticks were characterized by light to pale color and normally fed ticks are dark grey in color. Abnormally light color appearance of ticks was possible due to inability of ticks to access the deep blood vessel owing to immunological reaction at host skin. In this way ticks sucked extracellular fluid which is deficient of RBC's making ticks lighter in color (Fivaz *et al.*, 1991; Saran *et al.*, 1996). Protective effect of tick gut extract antigen was studied during a period of two years. Two groups of male Holstein-Friesian calves were made randomly, five animals in each group. The test group was inoculated three times with the adjuvant antigen. Control group was administered with Freund's adjuvant. Cattle were challenged with fifty pairs of homologous adult tick. Feeding and fertility of *H.anatolicum anatolicum* were recorded in immunized animals and compared with the unimmunized animals of control group. A significant reduction in percentage of engorgement, engorged weight, feeding index, percentage of oviposition, egg mass and fertility index are observed in the ticks fed on the test calves (Tabar *et al.*, 2004). In cattle humoral immune response was monitored to salivary gland, ovary and larval extracts by ELISA. Antibodies levels were monitored weekly for a period of nine weeks post infestation. An increase of the antibody level was observed after one week post infestation and reaches in a peak at 9^{th} week and decreased thereafter (Nikpay *et al.*, 2008).

2.2.3 Commercial vaccines

The development process of effective vaccines against tick is slow, despite its introduction as TickGARD and GAVAC to the market in 1994. Exposed antigen and concealed antigens are two sources which can be used as source of antigen in commercial vaccine development. A third group of antigens having properties of both exposed and concealed antigens, shows transmission-blocking and protective activity against a tick-borne pathogen (Nuttal *et al.*, 2006).

Friesian cattle were immunized with two inoculations of Tick-GARD vaccine and challenged 30 and 90 days later with *Boophilus annulatus* larvae, derived from 1.2g of eggs. No nymphs or adult ticks were found on the immunized cattle during four weeks after challenge. Repeated infestations (two to four) with larvae on three other calves during a period of 160 and 390 days after the immunization did not result in development of nymphal and adult stages. (Pipano *et al.*, 2003)**.**

2.2.4 Development of new vaccines

Vaccines are feasible tick control method that offers a cost-effective and environmentally friendly alternative to chemical control. Identification of tick-protective antigens remain one of the hurdles in vaccine development; both exposed and concealed are proven to be good candidates for vaccine development. Development of vaccines against multiple tick species is possible using highly conserved tick-protective antigens or by antigens showing immune cross-reaction to different tick species (de la Fuente and Kocan 2006). DNA vaccination of Merino crossbred sheep against *B. microplus* by usingBm86 full length gene was monitored. The antibody titer of the immunised animals was low but marginal decrease in mean engorgement weight of femaleticks was observed (De Rose *et al.,* 1999). We can manipulate tick endosymbionts and use them to control ticks by using chemotherapeutic, immunological and microbiological approaches. (Node *et al.,* 1997; Benson *et al.,* 2004). Immunological approach may be used to hit the endosmybionts of ticks instead of hitting vector (Shanmugam *et al.,* 1976).

3. Growing intestinal cell as monolayer

IDE8 is of Virginia isolate of *A. marginale* continuous cell line and is derived from embryonic source of *Ixodes scapularis.* They evaluated the potential of this organism for the use as an antigen for serologic tests and vaccines. The range of structural conservation of the major surface proteins (MSPs) between the cell culture-derived organism and the bovine erythrocytic stage was determined. Presently, *A. marginale* found in bovine erythrocytic is the source of *A. marginale* antigen (Barbet *et al.,*1999). IDE8 cell line was also used to propagate *Cowdria ruminantium* continuously for over 500 days by using the Gardel isolate from bovine endothelial cells as an inoculum. Infection of the tick cells was confirmed by PCR analysis, karyotyping, electron microscopy, and reinfection of bovine cells (Bell-Sekyi *et al.,* 2000). Lyme disease spirochete *Borrelia burgdorferi* was propogated to test ability of cell lines to phagocytose the agent. Cell lines used were ISE6 and IDE12 from *Ixodes scapularis* and DAE15 from *Dermacentor andersoni* Stiles. This study also showed that infection due to endosymbionts did not intervene significantly with the phagocytic activity of immunocompetent tick cells (Mattila *et al.*, 2007).

4. ***Summary***

Tick infestation is historical issue of livestock and other animal species in hot humid countries particularly in Asia (Pakistan, India, Bangladesh, Iran, Afghanistan etc.) and Africa. Ticks as vector can transmit a more pathogens as compared to any other arthropod. Ten % of currently known tick species act as vectors of pathogens in people and domestic animals. In Pakistan exotic cattle breed are more susceptible to ticks as compared to local e.i. Sahiwal and Dhanee

breeds. There are almost 900 species of ticks that are endemic to most continents and while feeding they can vector different bacterial, viral and bacterial diseases among the hosts. In order to devise control measures for tick infestation, it is necessary to conduct tick prevalence in the area. The use of acaricides was the commonest method for controlling ticks and ivermectin and trichlorfan are acaricides which were being normally used by the farmers. Uncontrolled and irrational use of acaricides is creating drug residue effects and selection of tick resistance against these chemicals. To mitigate the economical losses due to tick infestation it is necessary to work on the immunotherapy of ticks. Exposed (salivary gland) and concealed (midgut) antigens can be used for the preparation of tick vaccine. Different attempts have been made to immunize animals against the ticks by using crude extract. Tick GUARD and Gawac are two commercial vaccines available in the market for the control of ticks. Tick intestinal cell can be grown in cell culture as monolayer and further be harvested as source of antigen in vaccine preparation.

CHAPTER 3

MATERIALS AND METHODS

1. Collection and Morphological study of ticks

1.1 Study Area

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

1.2 Collection of Ticks and tick infestation level study

The ticks were collected in morning and evening in the months of July and August, 2007 from 710 cattle and 320 buffaloes. The tick collection and tick infestation level study was conducted with the help of field workers of Nestle Pakistan Ltd. Tick infestation rate and tick infestation level were estimated in all these bovine, 10 farms from each of three districts was selected randomly for these studies. Tick infestation level study was performed by categorizing cattle and buffalo population into three levels i.e. low, moderate and high infestation levels. Animals having 1-25 ticks were designated as low infestation level, while animals having 26-50 and above 50 ticks were characterized as moderate and high infestation levels, respectively. With the help of small forceps ticks were collected systemically as per Muhammad *et al.* (2008) starting from head towards

tail direction and placed in a Petri dish. Care was taken to avoid decapitation and shedding of legs. The tick samples were dispatched to Parasitology laboratory in clean and dry properly labeled plastic bottles. The mouths of these plastic bottles were covered by cheese cloth for proper aeration.

1.3 Tick collection for the T. annulata detection

Twenty morphologically speciated ticks were taken from each of five species (*Hyalomma anatolidum, H. dromedari, H.marginatum, Rhipicephalus microplus and Amblyomma variegatum*) and were used for detection of *T. Annulata* by PCR. Tick collection for this experiment was made in sterile glass bottles from cross bred cattle.

1.4 Identification of Ticks

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

1.5 Separation of female hard ticks from the male

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

2. Preparation of intestinal, salivary gland and whole homogenate tick vaccines and their inoculation into experimental animals

2.1 Source of ticks

Tick colony was established on a cross bred calf maintained in an experimental laboratory animal house, Department of Parasitology, University of Veterinary and

Animal Sciences, Lahore, with 30 ±3°C temperature and 75±5% relative humidity (Khan *et al.,* 1982). Partially engorged *Hyalomma* female ticks were collected from the calf and were used to make whole homogenate and organ based vaccines.

2.2 Salivary gland antigen isolation

Salivary glands were removed by the method of Walker *et al.* (1979). Partially engorged live female ticks were embedded on wax and salivary gland removed and stored at -40ºC until required. These glands were thawed and homogenized in phosphate buffer saline (PBS) as shown in Appendix-1, pH 7.2, using a sterile glass homogenizer. The resultant material was sonicated in 10-15 bursts of 30sec each with simultaneous cooling. Homogenate was then centrifuged 2000 revolution per minute (rpm) at 4ºC for 20min. The supernatant was used as Salivary Gland Extract (SGE) and stored at -40ºC until required.

2.3 Intestinal antigen isolation

Mid-gut was removed from semi engorged ticks as described by Das *et al.* (2000) during dissection and homogenized in 0.1M PBS, 1mM disodium Ethylene DTA, 0.02% merthiolate, pH 7.2, containing 1mM phenylmethane sulphonyl flourid (PMSF), in a glass homogenizer. The homogenate was sonicated four times for 30sec with interception of 10sec and then centrifuged at 14,000g for 10min at 4ºC. Supernatant was collected and pooled at -40ºC as intestinal tissue extract (ITE).

2.4 Whole homogenate antigen isolation

For whole homogenate vaccine ticks were cleaned up with 70% alcohol. Cleaned ticks were washed three times with normal saline to remove the debris, ground in tissue

grinder and filtered through muslin cloth to get the Whole Homogenate Tick Extract (WHTE). The WHTE was centrifuged at 6000g for 30min and supernatant was stored at -40ºC.

2.5 Protein Estimation of isolated antigens

The protein contentso f each SGE, ITE and WHTE was estimated by the method of Lowry *et al.* (1951) using protein estimation kit (Human, Germany: 157004).

Principle: Cupric ions react with protein in alkaline to form a purple complex. The absorbance of this complex is proportional to the protein concentration in the sample.

Reagent Composition:

Sodium hydroxide	200mM/L
Potasium sodium tartrate	32mM/L
Copper sulphate	12mM/L
Potassium oxide	30mM/L

Standard Solution:

Protein	8g/dL
Sodium azide	0.095%

Procedure:

1. Protein estimation was performed on spectrophotometer. The different materials are pipetted in cuvettes having one centimeter optical path by following pipetting scheme.

Reagents	Blank	Standard	Sample
Distilled water	20µl	-	-
Standard solution	-	20µl	-
Sample	-	-	20µl
Biuret Reagent	1.0ml	1.0ml	1.0ml

2. Above reagents were mixed thoroughly and allowed to stand for 10min at room temperature.

3. The absorbance (A) of the standard (St) and sample (S) were read at 546 nanometer against the blank.

Calculation:

Concentration of total protein (g/dl) = C = $\frac{A_{sample}}{A_{standard}} \times 8$

A_{sample} = Absorbance of sample $A_{standard}$ = Absorbance of standard

2.6 Preparation of oil based crude extract tick vaccines

Each of the oil based vaccine was prepared by mixing SGE, ITE or WTHE and Montanide ^{TM} ISA (Seppic: France) at 2:3 ratio. Each of the SGE and ITE vaccines contained 5mg protein/dose of the vaccine. Three types of vaccines each containing 5mg, 7.5mg and 10mg WTHE protein per 3ml of the dose, were prepared.

2.6.1 Safety test

The vaccines were injected at dose rate of 0.5ml to each of three rabbits for the confirmation of its safety.

2.6.2 Sterility

The vaccine was streaked on three different culture media (Thioglycerlate media, MacConkey agar and Tryptose blood agar). The media was observed for any growth after keeping them at incubation at 37^{o}C for 48 hours.

Experimental design for vaccination in rabbits

Twenty one healthy rabbits (6 months old) were selected and divided into 7 groups (Group I, II, III, IV, V, VI and VII), containing three rabbits in each group. Rabbits of group I, II and III were used to raise anti-serum against indigenous species of *Hyalomma, Boophilus* and *Amblyomma,* respectively. Rabbits of other groups were used to monitor antibody response against salivary gland, intestinal tissue and whole tick homogenate protein of *Hyalomma* species as shown in Table-1.

Table-1 Experimental design for vaccination in rabbits

	Cross-reactivity study amongst different genera (5mg/ml)			**Immune response of different organ based vaccine from Hyalomma species (5mg/ml)**			**Control group**
Group	**I n=3**	**II n=3**	**III n=3**	**IV n=3**	**V n=3**	**VI n=3**	**VII n=3**
Type of vaccine	OB-WHTE of *Hyalomma*	OB-WHTE of *Boophilus*	OB-WHTE of *Amblyomma*	OB-SGE	OB-ITE	OB-WHTE	Montanide based normal saline (pH 7.2).

OB-WHTE= oil-based whole homogenate tick extract

OB-SGE = oil-based whole salivary gland extract

OB-ITE = oil-based intestinal tissue extract

Rabbits of group I, II and III were primed intramuscularly with 0.5ml of OB-WTHE vaccine of indigenous species of *Hyalomma, Boophilus* and *Amblyomma* species, respectively, and boosted 21 days latter with the same route and dose. Rabbits of group IV, V and VI were primed (I/M) with 0.5ml of OB-SGE, OB-ITE, and OB-WTHE vaccine of *Hyalomma* species, respectively, and boosted 21 days latter through using same route and dose.

Experimental design for vaccination in Buffalo calves

Twelve healthy buffalo calves of age 18-24 months were selected at Buffalo Research Institute, Pattoki, Punjab and divided into four groups randomly, three animals in each group. Groups I, II and III were considered as experimental and group IV as control as shown in Table-2. These calves were being dewormed regularly and were maintained in similar husbandry conditions in the Institute. These animals were having no prior exposure to tick infestation and acaricides were being regularly used to control ticks and other ectoparasites.

Table-2 Experimental design for vaccination in buffalo calves

Group	Experimental groups			Control group
	I	II	III	IV
Antigen concentration	5.0mg/3ml	7.5mg/3ml	10.0mg/3ml	Montanide based normal saline (pH 7.2).

Each buffalo calf of all experimental groups was primed (3ml/dose) using intramuscular route and boosted with the same dose and route 28 days post priming. Amount of tick protein/dose was 5mg, 7.5mg and 10mg for each calf of group I, II and III, respectively. The control animals were inoculated with equal volume of the Montanide based normal saline (pH 7.2).

Blood sample of each rabbit of all groups and each buffalo calf was taken on 0, 15, 30 and 45 day post-priming. The serum was separated from each sample and transferred to each of the labeled vials and stored at -20^{0}C till required for antibody monitoring.

3. Monitoring sero-conversion of vaccinated rabbits and calves

Serum of each rabbit of each group was subjected to Agar Gel Precipitin Test ((Akhtar, 1995) to monitor the antibody response against each part (SGE, ITE and WTHE) of *Hyalomma* species and to monitor the cross reactivity among three genera of the hard ticks. The antibody titer in buffalo calves against tick extract was determined using Complement Fixation Test (CFT; Hudson and Hay, 1976). The anti-crude extract complement fixing antibody titer of each serum was determined.

3.1 Agar Gel Precipitation Test

Specific reactions in AGPT were indicated by precipitin lines between the tick antigen and the test serum. The results were compared with the identification of the reaction between the same antigen and that of the standard positive serum raised against tick antigen.

3.1.1 Optimization of AGPT

Optimization and standardization of the test was performed by using three different compositions of purified Noble agar. For this purpose 0.9gm, 1.0gm and 1.1 gm purified Noble agar was used in same weights (8gm) of sodium chloride and sodium azide (0.01gm) and same volume (100ml) of distilled water. It was found that gel was solidified optimally in that Petri dish in which poured molten agar with 0.9 gm purified Noble agar was used.

3.1.2 Preparation of agar gel plate

According to standardization results, 0.9% was prepared according to following composition.

Nobel Agar	(Oxoid: LP0028)	0.9gms
Sodium chloride		8.0gms
Sodium azide		0.01gms
Distilled water		100ml

All above three ingredients were weighed and mixed in 100ml dH_2O water and then heated to boil in microwave oven until a uniform suspension was obtained. The uniform suspension of the agar was then poured in to Petri dishes kept at room temperature and leveled surface and allowed to solidify. Approximately 70ml of gel in 100ml Petri dishes and 35ml in 60mm Petri dishes were poured. The thickness of gel was 4mm and solidified gel was transferred to a refrigerator till further used.

3.1.3 Punching of wells

Well guiding template of required well diameter got prepared from local market and was used for punching the wells in the agar gel. The diameter of the holes and the distance between the wells was 5mm, respectively. The lid of the plate was removed and the template was placed on agar gel plate. Care was taken that the template should not touch the surface of the agar gel plate. A metal gel borer was used to punch the wells through template in the agar gel. The punched gel in the wells was removed with great care using vacuum pump fitted with sterilized Pasteur pipette. The bottom of all wells was sealed by adding 20µl molten agar. This was done to minimize the leakage of antigen or antiserum between gel and glass plate.

3.1.4 Charging of wells

The known antigen (50µl/well) was added in three of peripheral wells and normal saline (50µl/ well) was added in 4th well, while unknown antiserum (50µl/well) was added in the central well. The plate was incubated for 72h in humidity chamber. The positive samples after thawing, were two fold diluted with PBS in microtiter plate with the help of pipette starting from the 1st well up to 10th of each dilution. All the two fold dilutions of positive samples were added to wells (50µl/well) to agar gel plate to monitor the antibody titer level. Appearance of the precipitin lines between the tick antigen and the serum sample within 72h was recorded.

3.2 CFT

In present study, CFT was carried out for the evaluation of tick vaccine as described by (Hudson and Hay, 1976), using Hyalomma whole tick homogenate suspension (WTHS) as antigen which was prepared in previous experiment and was stored at -40^{o}C for future use. In this test, test sera were raised in rabbits and experimental calves kept at Buffalo Research Institute, Pattokee. Oil based vaccines with 5mg, 7.5mg and 10.0mg per does were injected to these animals. Five percent washed sheep RBCs, rabbit anti-sheep erythrocytes antiserum containing sub-agglutinating level and Veronal buffer (Appendix-2) as diluents/stabilizer were used to conduct the test.

3.2.1 Amboceptor (rabbit anti-sheep erythrocytes antiserum)

Amboceptor (antibodies against sheep erythrocytes) were raised in rabbits by following the technique as described by Merchant and Packer (1983).

(a) Washing of sheep red RBC'S

I. A blood volume of 2ml was collected aseptically from the jugular vein of a sheep kept at Animal House of Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. An anticoagulant 5mg sodium citrate was added in 5ml sterile syringe before the collection of blood. Afterwards, the syringe containing blood was slightly agitated to mix the anticoagulant with the blood.

II. Washing of Red Blood Cells (RBC's) was performed with help of PBS. For this purpose blood was shifted to centrifuge tube and PBS was added to make total volume 10ml. The tube was centrifuged at 2000rpm for two min. The supernatant was discarded and PBS equal to packed cell volume (PCV) was added to the centrifuge tube carefully.

III. The packed RBC's were re-suspended in the saline by slight inverted motion of centrifuge tube and centrifuged at the same speed and time. The supernatant was again discarded and same quantity of PBS was again added. The process of RBC's washing was repeated thrice. Final volume of PCV 1.5ml was obtained and in this way 3.5ml PBS was added to get 10% suspension of the sheep erythrocytes. This suspension percentage of sheep RBC's was used in the rabbits to raise antiserum against it.

(b) Inoculation of washed RBCS to rabbits

Six rabbits of similar age and weight were purchased from local market and kept in experimental house for amboceptor raising. They were injected with prepared 10% sheep erythrocyte suspension as described in Table-3 .Three rabbits were used as un-inoculated controls.

Table- 3 Amboceptor raising schedule in rabbits

S. No.	Day of injection	Dose with route
1	1st	0.1ml: I/V of 10% sheep RBC's
2	3rd	0.2ml: I/V of 10% sheep RBC's
3	5th	0.4ml: I/V of 10% sheep RBC's
4	7th	0.6ml: I/V of 10% sheep RBC's
5	9th	0.8ml: I/V of 10% sheep RBC's
6	11th	2.0ml: I/V of 10% sheep RBC's

On day 21, the test and control rabbits were bled, their blood was collected directly from the heart separately and sera from each blood sample were separated and stored at -20^{o}C.

(c) Calculation of sub-agglutination titer

The serum samples were processed for titration of the amboceptor and determination of its sub-agglutination titer. A U-shaped, 96 well immuno-plates were used for the titration of the amboceptor.

1. Veronal buffer (50µl: pH 7.2, Appendix- 2) was added in each wells of three rows of the plate using multi-channeled micropipette.
2. Amboceptor (50µl) was added in all wells of first row except well No.12 and its two-fold dilution was made up to well No.11 discarding 50µl from well No. 11.
3. Washed sheep RBS's (50µl: five percent suspension) were added from well 1 to well 12 and the plate was incubated at the room temperature for 30 min.
4. Both the test and the control rabbit sera were monitored for the amboceptor titeration.

The general plan for determination of agglutination level of the amboceptor is presented in Appendix -3.

The highest dilution of the rabbit serum showing agglutination was considered as 1 HA unit titer. Sub-agglutination level of amboceptor i.e., the 50% dilution of 1 HA unit titer of the amboceptor was used for sensitization of 10% washed sheep RBS's.

(d) Sensitization of sheep RBC's

1. The sub-agglutination level of the amboceptor (10ml) was taken and treated at 56^{o}C for half an hour in water bath to inactivate the rabbit complement and then added to 10ml of the 10% washed sheep RBC's suspension.

2. This mixture was incubated at 37^{o}C for 20min for sensitization of sheep RBC's.

3. The mixture was centrifuged at 2000rpm for two min and the supernatant was discarded and the pellet of the RBC's was re-suspended.

4. The centrifugation process was repeated thrice by re-suspending the sensitized sheep RBC's in the saline.

5. Finally 0.2% suspension of the sheep RBCs was made (0.2ml of the sensitized R BC's was added in 99.8ml of the saline) to be used for titration of the complement.

(f) Complement

Complement was used as indicator in hemolytic system in CFT and serum from guinea pig was used as complement source as guinea pig serum contains a greater concentration of all complements than that of other animals (Merchant and Packer, 1983). Four guinea pigs were kept at the Experimental house of Department of Parasitology, University of Veterinary and Animal Sciences, Lahore.

Before bleeding, guinea pigs were kept under air conditioned environment for overnight. Each guinea pig was sacrificed and bled in large beaker. Serum was collected after coagulation of blood and its aliquots of two micro liters were made in Eppendorf tubes. This serum was used as source of complement.

3.2.2 Calculation of hemolytic units of guinea pig serum

Four hemolytic units (4 HL) of guinea pig serum were calculated and U-shaped 96-well micro titration plate was used for titration of complement. Following protocol was used:

1. Using multi-channeled micropipette, 50µl of PBS was added from well No.1 to well no.12 in all rows.

2. A 50µl amount of the guinea pig serum was added in the first well and two-fold dilution up to well No.10 was made of first row.
3. The 50µl the sensitized sheep RBC's (0.2%) were added from well 1 to well no. 12.
4. Well number 12 was kept as negative control. Plate was incubated at 4ºC for overnight.

The plane for calculation of 4-Hemolytic unit is presented in Appendix-4

Positive= Lysis= Clear suspension of the sensitized sheep RBC's

Negative= No lysis/button formation=sedimentation of the sensitized sheep RBC's

Procedure of CFT

The CFT was performed as recommendation of Merchant and Packer (1983). Brief steps are as follows:

1. An amount of 50µl PBS solution was added from well number 1 to well number 12 of the immunotitration plate.
2. The 50µl of the immune serum was added in well no. 1 and its two-fold dilutions were made up to well number 9.
3. The 50µl of tick antigen (whole tick extract) was added from well number 1 to well number 10.
4. Then 50µl of the complement containing 4 HI units was added from well number 1 to well number 11 and the plate was incubated at 37ºC for ten min.
5. Finally, 50µl of the sensitized sheep RBCs (0.2%) was added from well number 1 to well number 12

In this way each of the immune serum sample (test serum) and control serum samples was titrated against tick antigen.

Statistical Analysis:

The geometric mean titer (GMT) of antibody data of AGPT and CFT was calculated by the technique described by Villages (1998).

In case of AGPT it is highest dilution of the serum showing Agar Gel Precipitation lines against specific antigen, while, it the highest dilution of no hemolysis in quantitative assay in Complement Fixation Test. \

The formula of GMT is as follows:

$$GMT = \frac{\text{antilog}\,(\log x_1 + \log x_2 + \log x_3 \ldots\ldots + \log x_n)}{n}$$

n= no. of samples

Geometric mean titer of each group at different time post-priming was analyzed through Kruskal-Wallis Test as described by Choudhry and Kamal (2006).

Suppose, antibody titer levels in eight wells are 7,6,6,7,6,5,7 and 7.

Now, frequency is $5\times1=5$

$6\times3=18$

$7\times4=28$

Total=51 n=8

51/8= 6.37

So, GMT=$\log_2 6.4$

The general plan for calculation of anti-tick immunogen- complement fixing antibody units is shown in Appendix-5.

4. Monitoring capacity of Hyalomma whole homogenate tick vaccine in controlling tick infestation in crossbred animals by ELISA

Twelve crossbred male calves of same age (6-9) and weight were selected from Aslam Gujjar Livestock Farm, Lahore. These animals were thoroughly examined for tick infestation and found free of ticks. These calves have no previous exposure to tick infestation because acaricides were regularly used in these animals. The animals were divided randomly into two groups, six animals in each group. Group I was assigned as experimental control and group II was assigned as control.

4.1 Vaccination of crossbred calves with Hyalomma whole homogenate tick vaccine

Crossbred calves of group I (calf no. AGF-1, AGF-2, AGJ-4, AGF-10, AGF-19, and AGF-20) were kept as control. Crossbred calves of group I1 (calf no. AGF-9, AGF-12, AGF-14, AGF-22 AGF-23 and AGF-24) were vaccinated with oil-based *Hyalomma* whole homogenate tick vaccine with 7.5mg/dose antigen concentration.

Table-4 Schedule of immunization of *Hyalomma* whole homogenate vaccine in crossbred calves

Immunization	Time(days)	Group II (non-vaccinated)	Group I (vaccinated)
Primary	0	3 ml of PBS(pH7.2)+adjuvant	3 ml of oil based *Hyalomma* whole homogenate tick vaccine
Booster	21	3 ml of PBS(pH7.2)+adjuvant	3ml of oil based *Hyalomma* whole homogenate tick vaccine
Booster	42	3 ml of PBS(pH7.2)+adjuvant	3ml of oil based *Hyalomma* whole homogenate tick vaccine

4.2 Challenge infestation

Challenge infestation with 100 adult female *Hyalomma anatolicum* was performed after 15 days of first booster inoculation in both the groups. Cotton bags of coarse material were used to restrict the escape of the ticks by following the method of Bhattacharyulu *et al.* (1975). All the animals in experimental group as well as control group were observed daily to see the number of ticks attached and dropped.

The rejection percentage of adult female ticks was calculated as under:

$$\text{Rejection percentage} = \frac{\text{Number of dropped adults female}}{\text{Number of adults released}} \times 100$$

Dropped ticks were kept at 29ºC and 85% RH (Andreotti *et al.,* 2002) for three weeks of incubation period and weight of laid egg was measured. The dropped ticks were keenly observed and classified into abnormally (white or pale yellow in color) and normally fed (dark grey color) ticks according to their appearance. Differences in rejection percentage, engorgement period, engorgement weight, egg mass and reproductive index in immunized and control group were calculated statistically by Student-t test to find the efficacy of the vaccine in experimental group and control group.

Blood samples from cross-bred calves were collected on day 0, 15, 30, 45, 60, 75, 90, 105 and 120 and serum samples were stored at -20ºC until used. Antibody response in controlled and vaccinated group was determined by Indirect Enzyme Linked Immunosorbant Assay (ELISA).

4.3 Indirect ELISA

The ELISA was performed for detection of antibodies against antigen prepared from whole tick homogenate (Ismael *et al.,* 2003). For this purpose horse radish peroxidase conjugated antibovine IgG (Sigma- Aldrich: Cat # A 8917) in rabbits as conjugate and 'O' phenylene diamine (OPD) with hydrogen peroxidase in citrate phosphate buffer as substrate were used. Incubation period for antigen and conjugate concentration were optimized by Checker board titration method and following testing procedure was performed:

4.3.1 Preparation of coating buffer

50 mM Carbonate buffer (pH 9.6) was prepared for coating the antigen to ELISA plate. (Appendix-6)

4.3.2 Preparation of washing buffer

The washing buffer (0.05% Tween 20) was prepared by adding 0.5ml Tween20 in 1000ml Phosphate buffered saline (Appendix-7).

4.3.3 Standardization of solid Phase

Three types of 96 well microtiter plates (U bottom, Flat bottom and flat bottom EIA) were used for the coating of antigen.

4.3.4 Preparation of substrate

Substrate was prepared by dissolving 30mg OPD tablet in 30µl of 30 % H_2O_2 v/v in 75 ml citrate buffer (Appendix-8).

4.3.5 Coating of plate with tick antigen

Plates were coated with tick antigen prepared in previous experiment. Different concentrations of this antigen ranging from 25-200ug/µl were prepared in coating buffer.

4.3.6 Dilution of conjugate

The conjugated Antibovine IgG in rabbit (A 8917:Sigma Aldrich) was reconstituted and its 1:2000 dilution was made in solvent (BSA 4%+PBS 1X). Aliquots of the solution were prepared and stored at 4^{o}C till further used.

4.3.7 Dilution of serum samples

The serum samples were diluted 1:100 with diluent (BSA 4%+PBS 1X).

Protocol for Indirect ELISA:

1. Plates were coated with 100µl of diluted antigen/well and kept at 4^{o}C for overnight. The plates were covered with adhesive plastic sheet to prevent evaporation of antigen. For each dilution of antigen two rows of plates were used.

2. Next day the plates were washed three times with washing buffer (1X PBS +Tween 0.05%) 300µl per well

3. Blocking (2% bovine serum albumin) solution was added in volume of 150µl/ well and then plates were incubated for one hour at 37ºC.

4. The plate were emptied and washed with PBS Tween 20 washing buffer.

5. Two fold serial dilution of serum samples were made in PBS. From each dilution 100µl of serum/ well was dispensed from well 1-10 in each row. Plates were incubated for one hour at 37ºC.

6. Plates were washed three times with washing buffer.

7. Conjugate was added in all wells (100µl/well) and plates were incubated at 37ºC for two hours.

8. Substrate ('O' phenylenediamine with Hydrogen peroxide in citrate buffer) was added to all wells (100µl/ well) and was incubated at 37ºC for 30 minutes.

9. After 15min the reaction was stopped by adding 100µl of NaOH (0.2M).

10. Observations were recorded on ELISA reader at 405nm wavelength.

5. Detection of T.annulata by PCR

5.1 Processing of samples for DNA extraction

DNA was extracted from ticks by Phenol-Chloroform method as described by D'Oliveira *et al.,* 1997. In brief, ticks were removed from ethanol (70%) and dissected into two halves with the help of sterile blade. New blade was used for each tick dissection. Both halves of the dissected ticks were boiled in 1.5ml microcentrifuge tube in 200µl PBS for

10min. One percent SDS was added to 150µl boiled tick sample which was removed thereafter with phenol: choloroform (1:1) and phenol: isoamyl alcohol (24:1), respectively. Two micro liters of extracted DNA was used for PCR reaction. DNA quantity was measured at 260 nm. The ratios of 260/280 were ranging from 1.80 to 1.95 for purified DNA samples which showed that preparations were free of any major protein. After quantification, DNA samples thus extracted were stored in -40ºC till used for PCR analysis.

5.2 Preparation of final reaction volume and PCR analysis

The mixture for PCR was prepared in volume of 50µl containing 200uM of dNTPS, 1.5mM of $MgCl_2$, 10mM of Tric HCl, 50mM of KCl, 200pM of each of reverse and forward primer, 0.1% of Tritone and 2.5IU units of Taq DNA polymerase. The temperature cycles used for PCR analysis are: initial heating for five minutes at 94ºC, following by 35 cycles of 94ºC for 30sec, 55ºC for 30sec and 72ºC for one minute. Final extension step was programed at 72ºC for seven minutes (Sirigireddy and Ganta, 2005). Two controls, one negative and one positive were included in each test. The reaction mixtures were performed in automatic conventional DNA thermocylcler (Esco Technologies, Inc.: USA).

5.3 Primers

Primers (IA-1 and IA-2: First base, Singapor) were used for the amplification of the gene coding for surface protein of the merozoite (Table-5).

Table-5 Oligonuleotide primers used for amplification in PCR Analysis

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

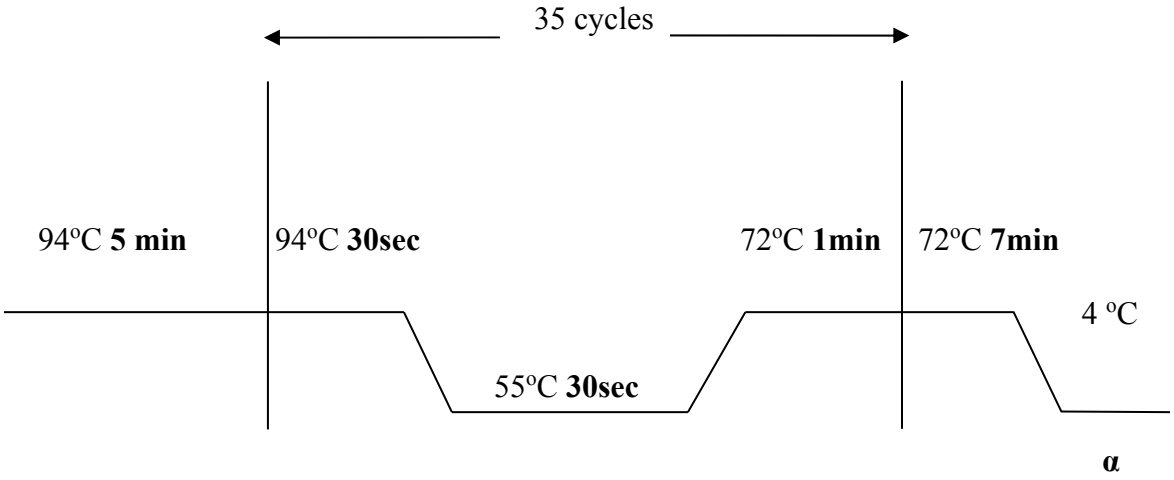

Figure-1 PCR thermal conditions for *T. annulata primers* (I-A1/I-A2)

One negative control of PBS and one positive control were included in the PCR Analysis.

5.4 Electrophoresis

The amplified samples were separated on electrophoresis containing 1.0% Agrose Gel (Research Organics, Inc. USA). 1X Tris-acetate EDTA buffer was prepared from 50X concentration and 50m1 of this was taken in a 200ml flask. Agarose Gel (0.5gm) was added in it and heated the gel in microwave oven for one minute. One µl of 10mg/ml ethidium bromide was added and mixed in the flask. The molten gel was poured into gel

plate (with inserted comb) and allowed to solidify for 20min at room temperature. Two hundred and fifty milli liters prepared 1X Tris-acetate EDTA buffer as dissolving medium was poured in the gel tank and five micro liters of ethidium bromide was also added in it.

Bromopehonl blue (Fermentas: Europe) was added as loading dye in the PCR product. For this purpose, 2µl of 6x Bromophenol blue was added in 5µl of PCR product and mixed thrice by micropipette. One Kb plus DNA ladder and λ hindIII were run along with PCR product for the detection of any non specific reaction and to compare the size of band. The bands were monitored under UV light and after proper cropping of the images the data was saved for record keeping.

6. *Growing of intestinal epithelial cells of hard tick as monolayer*

6.1 Preparation of culture medium

Cell culture medium of required concentration (13.99mg/L) was prepared using 1X L-15 (MP: Biomedicals, LCC: USA) as insect medium to grow tick intestinal cell (Bel-sekyi *et al.,* 2000). The prepared combined medium was kept in incubator for 48h at 37^{o}C and checked for any growth.

6.2 Surface sterlization of Hyalomma ticks

Ten semi-engorged adult female *Hyalomma* ticks were collected from tick colony and sterilized by dipping and intermittent shaking in 0.5% benzalkonium chloride solution. The ticks were then washed with 70% alcohol and rinsed in three changes of sterilized distilled water.

6.3 Isolation of intestinal cell

After surface sterilization ticks were dissected aseptically and intestines were removed and placed in culture medium placed in small Petri dish. Suspension medium was filtered through muslin cloth. For breaking the gut into cells filtrate was mixed thereafter on hot plate stirrer by keeping heat knob off for 15min. These broken cells were shifted to Karrel flask (25ml) in safety cabin.

6.4 Examination of tick intestinal cell viability

A solution with following composition was prepared for the examination of tick intestinal viability under compound microscope.

1. Cell suspension (from Karrel flask) 0.2ml
2. Basal salt solution 0.4ml
3. Typin blue (4%) 0.4ml

A drop from above solution was poured on hemocytometer slide and examined under inverted microscope for viability. Afterwards cell culture flask was kept in incubator with 5% CO_2 at 37^oC and media was changed regularly after 3-4 days.

6.5 Examining confluence rate of tick intestinal cells

For the examining the confluence rate, T-25 flask was scratched and with the help of pipette and 200µl volume of cell suspension was shifted to micro centrifuge tube. The tube was centrifuged at 400rpm for 2min and one drop from bottom was taken on glass slide. After air drying the slide, it was stained by using Hema staining kit for which following reagents with given duration of staining were used:

1. Hema-3 (fixative solution) 20sec
2. Hema-3 Solution 1 7sec
3. Hema-3 Solution 2 4sec

After staining the slide, it was rinsed with tap water and allowed to air dry at room temperature. Examination of confluence percentage was examined under oil immersion lens under light microscope.

CHAPTER 4

RESULTS

1. Tick prevalence in cattle and buffaloes

This study was conducted in the months of July and August, 2007. Temperature and humidity during study period were 30-35^{0}C and 60-70%, respectively (Appendix-9). In all three districts *Hyalomma species* were found the highest in prevalence at 61% as compared to other species (Table-6, Figure-5). Faisalabad district was the highest for *Hyalomma species* being 43% followed by Jhang at 28% and Khanewal at 29%. *Rhipicephalus (Boophilus) species* were the second most prevalent species with an average prevalence of 28%, Faisalabad being the highest at 66% followed by Jhang at 26% and Khanewal at 7%. *Amblyomma species* were the third major species detected (average=eight percent). This tick species was the highest in Jhang at 86% compared to Faisalabad at eight percent and Khanewal at six percent. *Haemaphysalis species* were the lowest at three percent in prevalence in three districts; Jhang being the highest at 58% followed by Faisalabad at 18% and Khanewal at 24%. No *Rhipecephalis* (*Boophilus*) *species* other than *R. microplus* was detected in these districts in our study. In sex-wise distribution of hard ticks, adult female ticks were higher as compared to adult male ticks and they were at 85% and 81% for *Hyalomma species* and *Amblyomma species*, respectively. *Rhipecephalis (Boophilus) species* and *Haemaphysalis species* have similar female to male ratio as 77% of adult ticks were females.

Table-6 Genus-wise hard tick distribution in cattle and buffaloes in three districts of Punjab

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

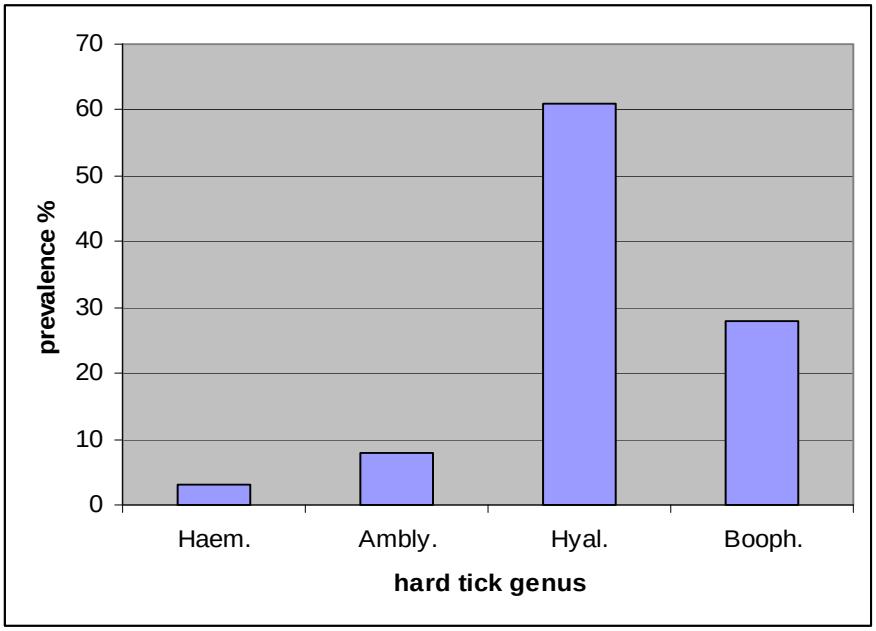

Figure-2 Overall prevalence of different hard tick genera in Faisalabad, Jhang and Khanewal districts

Tick infestation rate in cattle and buffalo population were also estimated in the districts and compared. It was found that infestation rate was higher in cattle at 70% as compared to buffalo population at 34% (Table-7). Tick infestation level study showed that high

infestation level (tick number above 50) was 59% in cattle population as compared to 18% in buffalo population. Moderately infestation level (tick number 25-50) was 38% in buffalo, white it was 23% in cattle. In low level infestation (1-25), buffaloes have higher at 44% as compared to 18% in cattle.

Table-7 Tick infestation levels comparison between cattle and buffaloes

Species of bovine	Tick Infestation level (%)		
	Low	Moderate	High
Cattle	18	23	59
Buffaloes	44	38	18

Low level infestation=1-25 Moderate level of infestation=26-50 High level of infestation=above 50

2. Preparation of different whole homogenate oil based vaccines

Each of the hard tick species when ground in pestle mortar and mixed in phosphate buffered saline (PBS: pH 7.2), resulted into dark brown suspension. The whole tick suspension when ground in tissue grinder and filtered through muslin cloth resulted into light brown suspension. Similarly, each of the mid gut and salivary gland of each of the whole tick when separated, admixed and sonicated in the PBS, resulted into light colored transparent solution. Whole tick homogenate, mid gut and salivary gland suspension contained 17.3, 25, and 24 mg/ml of the protein. Each of the protein suspension when admixed with Montanide (ISA-70) resulted into milky white homogenous suspension.

3. Monitoring sero-conversion of vaccinated rabbits and calves

Each of the whole tick homogenate vaccine prepared from either of the species of *Hyalomma, Rhipecephalus (Boophilus)* or *Amblyomma*, when injected to rabbits (0.5 ml: intramuscularly) induced detectable level of anti-whole tick homogenate protein agar gel precipitating (WTH-AGP) antibodies. Each antiserum containing each tick specific WTH-AGP antibodies cross reacted with tick specific homogenate protein antigen (Table-8). The proteins of each of the hard ticks were antigenically similar. In the same way, vaccine prepared from either salivary gland, mid-gut or whole tick homogenate protein of the same species of *Hyalomma* induced detectable anti-tick organ specific protein agar gel precipitating (TOSP-AGP) antibodies (Table-9). The vaccine prepared from salivary gland induced 0, 3.2 and 6.5 geometric mean titer of TOSP-AGP antibodies on 0, 15 and 30 days post priming, respectively. The vaccine prepared from mid gut protein induced 0, 6.5 and 13.0 geometric mean titer of TOSP-AGP antibodies on 0, 15 and 30 days post priming, respectively. The vaccine prepared from WTHE protein induced 0, 13 and 26 geometric mean titer of TOSP-AGP antibodies on 0, 15 and 30 days post priming, respectively. The WTH vaccine induced higher titer of the antibodies in rabbits as compared to that of salivary gland and mid gut protein vaccines.

Table-8 Cross reactivity among species of *Hyalomma, Rhipicephaus* (*Boophilus*) and *Amblyomma*

Rabbit Anti-serum against Whole Tick Homogenate	Whole Tick Homogenate Antigen		
	Hyalomma species	***Rhipicephalus (Boophilus) Species***	***Amblyomma species***
Hyalomma species	+	+	+
Rhipicephalus(Boophilus) species	+	+	+
Amblyomma species	+	+	+

Table-9 Comparative antibody response of rabbits to oil based vaccine prepared from different parts of the *Hyalomma species*

Days post priming n=3	Group I (salivary gland) n=3			Group II (mid gut) n=3			Group III (whole tick) n=3			Group IV (control) n=3		
0	0	0	0	0	0	0	0	0	0	0	0	0
15	2^2	2^2	2^1	2^3	2^2	2^3	2^4	2^3	2^4	0	0	0
30	2^3	2^2	2^3	2^4	2^3	2^4	2^5	2^4	2^5	0	0	0

unit of antibody=AGPT units

Group I : Oil based vaccine of the tick salivary gland protein (5mg/3ml).

Group II : Oil based vaccine of the tick mid gut protein (5mg/3ml).

Group III: Oil based vaccine of the whole tick homogenate protein (5 mg/3 ml).

Group IV: Oil based vaccine of the phosphate buffered saline without tick protein.

Effect of WTH protein amount per dose of the vaccine on the antibody response of buffalo calves was monitored by Complement Fixation Test (Table-10, Figure-7). The vaccine containing 5 mg protein /dose did not induce antibody response in buffalo calves. However, the vaccine containing 7.5 mg protein/dose induced 32, 39.4 and 13.0 GMT of WTH-CFT antibodies on 15, 30 and 45 days post priming, respectively. The vaccine containing 10 mg protein/dose induced 32, 64 and 19.7 GMT of WTH-CFT antibodies on 15, 30 and 45 days post priming, respectively. It was further noted that antibody response of buffalo calves to vaccine containing 7.5mg protein was not significantly higher that that vaccine dose containing 10.0 mg protein *(P>0.05)*. It means that the WTH vaccine containing more than 7.5 mg protein per dose can be used to induce detectable level of WTH-CFT antibodies.

Table-10 Antibody response of buffalo calves to hard tick homogenate vaccine

Days post-priming	**Anti-Hard Tick Homogenate Complement Fixing Antibody Titer**			
	Group I (n=3)	**Group II (n=3)**	**Group III (n=3)**	**Group IV (n=3)**
0	0 , 0, 0 (0)	0 , 0, 0 (0)	0,0, 0 (0)	0, 0, 0 (0)
15	0, 0, 0 (0)	2^6, 2^5, 2^4 ($2^{5.0}$)	2^6, 2^4, 2^5 (2^5)	0, 0, 0 (0)
30	0, 0, 0 (0)	2^6, 2^5, 2^5 ($2^{5.3}$)	2^7, 2^6, 2^5(2^6)	0, 0, 0 (0)
45	0, 0, 0 (0)	2^4, 2^4, 2^3 ($2^{3.7}$)	2^5, 2^4, 2^4($2^{4.3}$)	0, 0, 0 (0)
CGMT	2^0 **a**	$2^{4.6+0.9}$ **b**	$2^{5.1+0.9}$ **b**	2^0 **a**

Figures in parenthesis indicate geometric mean titers (GMT) and CGMT mean cumulative geometric mean titer. The values with similar letter are not significantly different *(P>0.05)*.

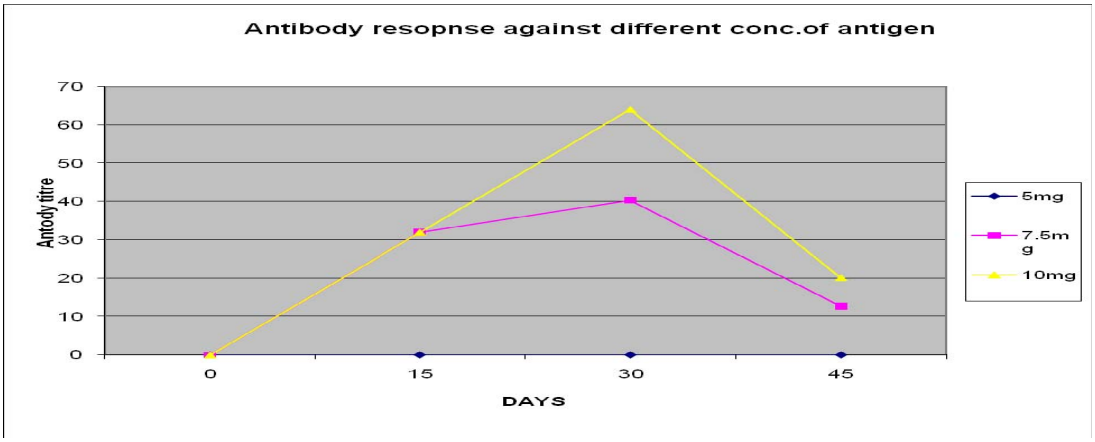

Figure-3 Immune response of buffalo calves against *Hyalomma* whole homogenate vaccines with different concentration of antigen

Unit of Antibody is WTH Complement Fixation Test antibodies.

4. *Monitoring efficacy of Hyalomma whole homogenate vaccine in Crossbred animals*

We performed two experiments to evaluate the Hyalomma whole homogenate tick vaccine in Crossbred calves. In one experiment we observed the effect of vaccine in the vaccinated Crossbred calves after giving artificial tick infestation. In second experiment we measured the immune response of vaccination in vaccinated and non-vaccinated Crossbred calves through ELISA.

In immunized group, small nodule and medium exudation were observed at attachment sites as inflammatory signs. In non-immunized group, no such observation was observed. In immunized animals, rejected adult female ticks were white to pale yellow in color due to the lack of red blood cells intake in their meal. Rejection percentage mean in immunized group was significant higher ($p<0.05$) as compared to control group. There was no significant difference ($p>0.05$) of engorgement period between immunized and control group.

Table-11 Effect of whole homogenate *Hyalomma* tick vaccine on feeding and development performances of Hyalomma anatolicum in Crossbred calves. (The values are expressed in mean±S.E.)

Variables	**Group-1 (non-immunized)** n=6	**Group-11 (immunized)** n=6
Rejection percentage	68.8±1.9	38.3±1.5
Engorgement period (days)	6.7±0.3	6.0±0.6
Engorgement weight (mg)	151.7±4.4	226.7±8.8
Egg mass (mg)	83.0±2.6	304.7±3.2
*Reproductive index	0.5±0.02	1.3±0.06

*Reproductive index: Mean egg mass weight/Mean engorgement weight

Engorgement weight of immunized group was significantly lower ($p<0.05$) as compared to immunized group. Immunization resulted into significantly decrease ($p<0.05$) egg mass production as compared to controlled group. Reproductive index in immunized group was significantly lower ($p<0.05$) as compared to control group. These parameters showed that oil based Hyalomma whole homogenate tick vaccine gave significant levels of protective immunity (Table-13).

Table-12 Comparison of immune response of tick vaccine between vaccinated and non-vaccinated Crossbred calves through ELISA (Antibody titer expressed in geometric mean and geometric SD)

Days Post-vaccination	**Vaccinated Group**	**Non-vaccinated group**
0	360±7	362±6
15	367±7	282±4
30	1448±6	362±5
45	1911±5	760±6
60	2353±4	662±5
75	3327±3	452±6
90	2230±3	382±4
105	1833±4	352±7
120	1511±5	366±6

Each serum sample was diluted 1:100 and was serially diluted two fold 1:100 to

1: 51,200. In the study mean antibody responses of the vaccinated group rose throughout the course of study whereas the mean antibody responses of control group remained relatively constant. Antibody titers were measured by using sera drawn before vaccination from the animals of vaccinated group and control group. The mean antibody levels of each group of cross-bred calves before vaccination was same. The antibody level o of immunized group was significantly higher ($P<0.05$) than that of non-immunized group.

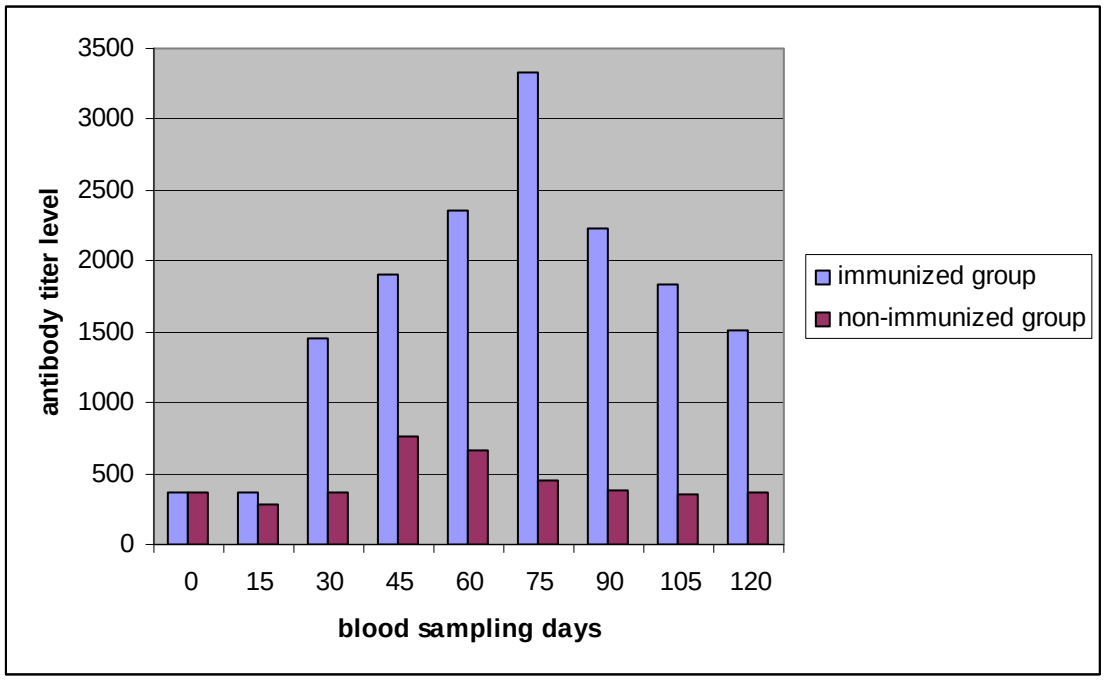

Figure-4 Comparison of antibody titer level between cross bred calves immunized with whole homogenate *Hyalomma* vaccine and non-immunized crossbred calves

5. Detection of T. annulata in hard ticks by PCR

Amplification:

Thermocycler was used to perform amplification for 30 cycles at different cyclic conditions. It was revealed that primers IA-1/1A-2 anneal at Tm 60 ^{o}C.

Gel electrophoresis:

Amplified PCR product was analyzed on gel electrophoresis by using 1.0 %

agrose gel under U.V. light.

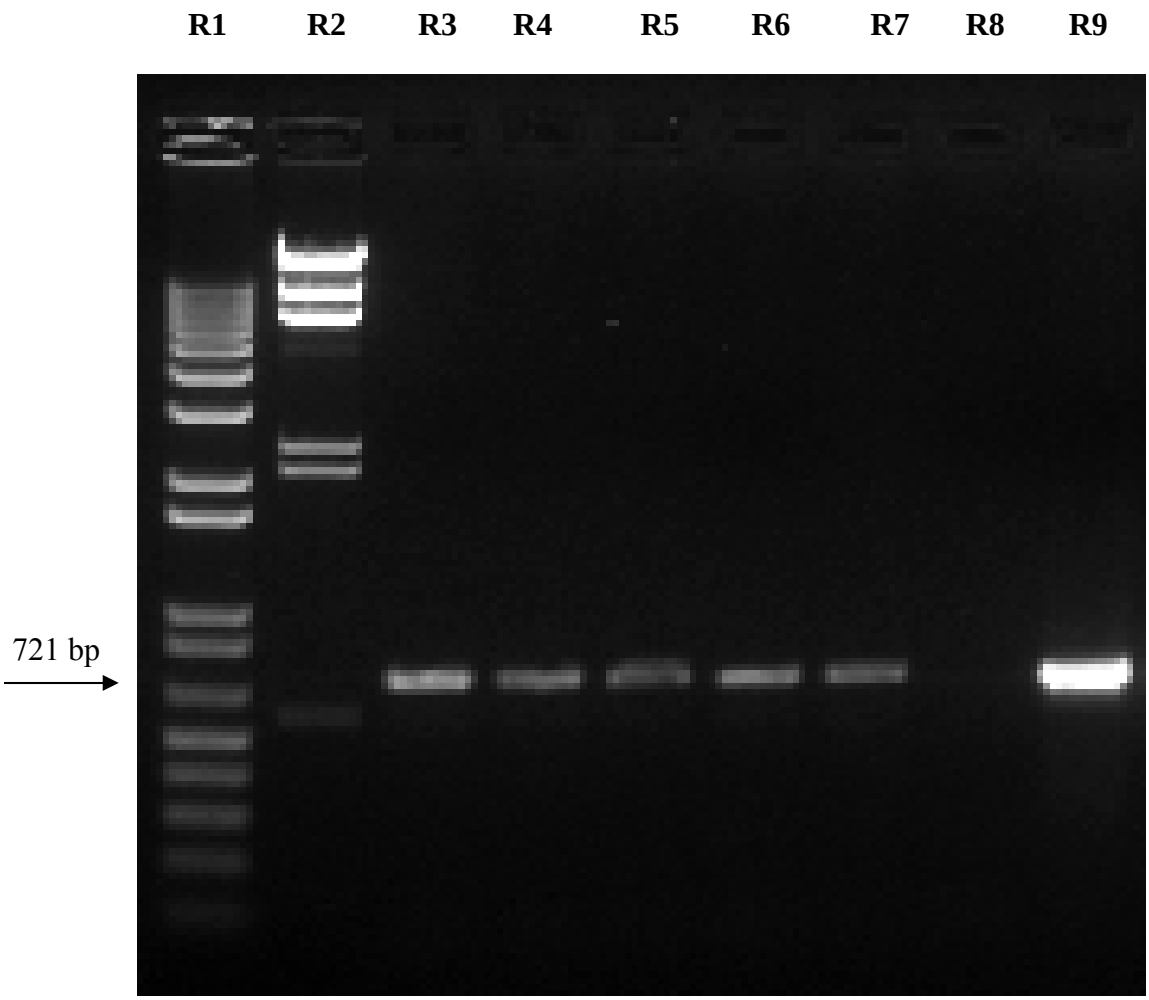

Figure-5 Analysis of amplified products of samples taken from ticks with primers AI-1/IA-2

R1= 1Kb + DNA ladder
R2= λ hind III
R3, R4, R5, R6, R7= positive samples of *Th. annulata* (IA-1/ IA-2)
R8= negative sample
R9= positive control

T. annulata was detected in different *Hyalomma* species by PCR using primer set IA-1/IA-2. The 721-bp band was generated in all positive samples tested with IA-1/IA-2. The specificity of PCR was tested by using positive control samples.

PCR based detection of *T. annulata* was performed from 100 ticks randomly selected to represent the species of ticks (20 ticks per tick species). The prevalence of *T.annulata* in *Hyalomma anatolicum* and *H.dromedari* ticks was 50% and 40% respectively. No theilerial organism was detected from *H. marginatum, R.annulatus* and *Amblyomma variegatum* ticks.(Table-11).

Table-1 3 Prevalence of *T. annulata* in different hard tick species by PCR Analysis

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

Specificity of the PCR:

The specificity of PCR test was examined by positive control samples by were provided from Theileriosis project funded by Nestle Pakistan.

6. Growing of intestinal epithelial cells of hard tick as monolayer

After overnight incubation at 37 ^{0}C, Karrel flask was placed under inverted microscope and examined for the evidence of cell growth. It was found that intestinal cells were growing as under inverted microscope spindle shaped cells were found which the signs for cell division are.

CHAPTER 5

DISCUSSION

1. Prevalence study

Faisalabad, Jhang and Khanewal are three closely located districts in the Punjab province of Pakistan, which is also known as Central Punjab. Central Punjab is canal irrigated area and has fertile land which supports agriculture and livestock farming. We selected Faisalabad, Jhang and Khanewal as these districts are considered as top livestock population districts in Pakistan. In the region people live in rural areas and depend upon crop agriculture and livestock for their livelihood. These districts have similar weather conditions around the year (Fig-6, Fig-7 and Fig-8) as they are located closely in hot and humid Central Punjab (Fig-9).

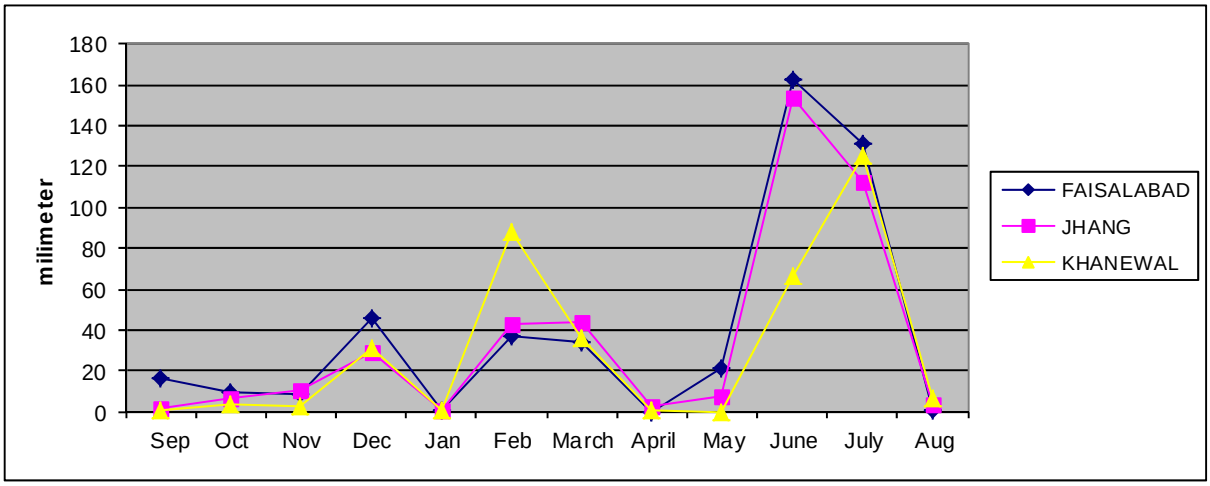

Fig-6 Monthly rain fall in three districts of Punjab

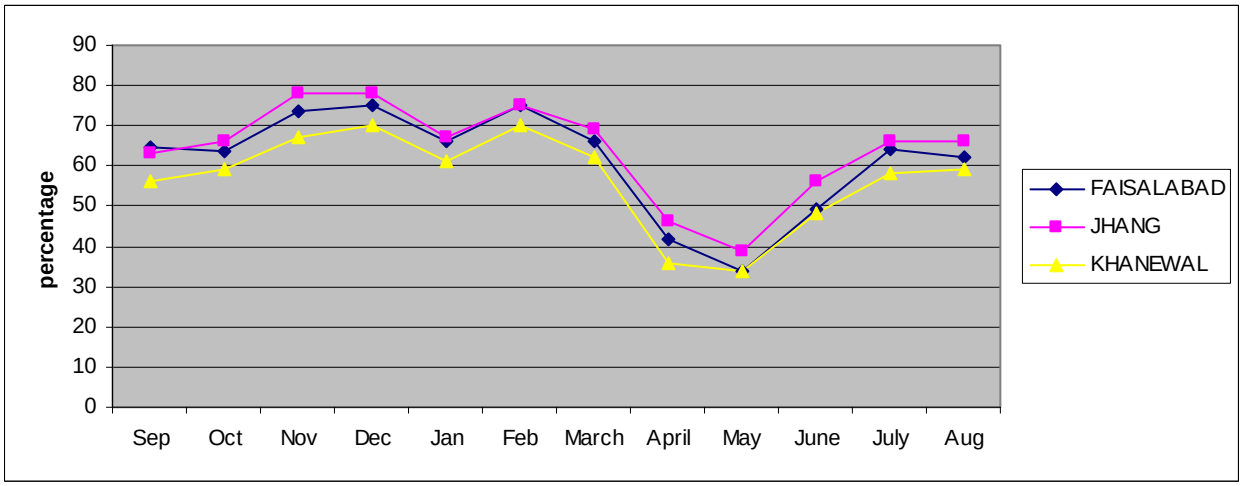

Fig-7 Mean Monthly humidity in three districts of Punjab

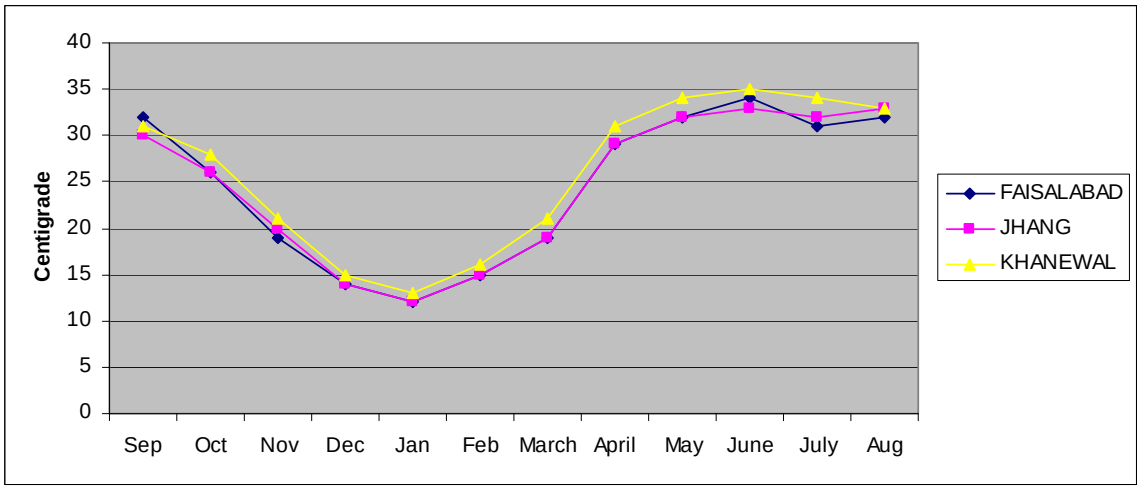

Fig-8 Mean monthly temperature in three districts of Punjab

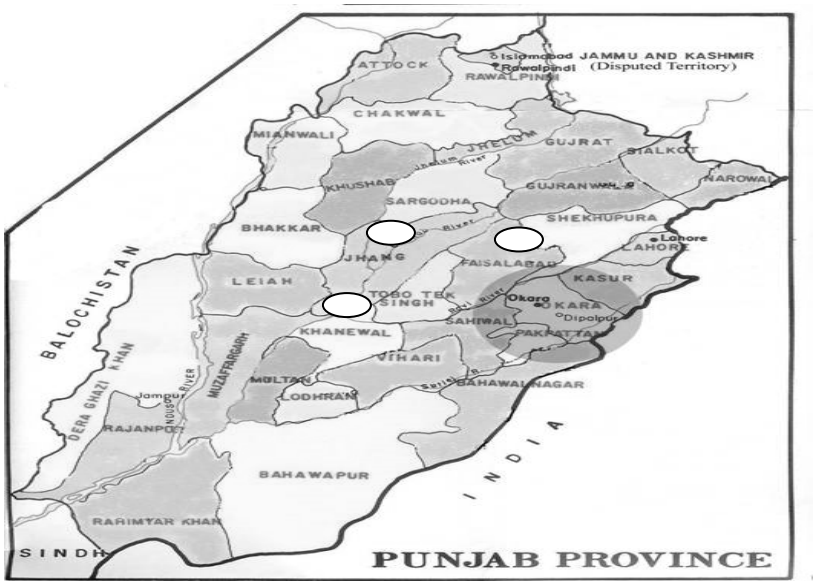

Figure-9 Map showing tick collection districts (Faisalabad, Jhang and Khanewal)

Tick infestation prevalence in these areas is high due to favorable humidity and temperature conditions, particularly during hotter months of a year. Reported incidences of tick borne diseases such as theileriosis may be the indicator of high tick infestation in these districts (Khan *et al.*, 2004).

Tick distribution studies have been performed in different areas of Pakistan to evaluate tick burden and their economic impact on livestock farming (Table-5). In one study which was performed by Durrani and Shakoori, 2009 in Rawalpindi, Lahore and Multan districts of Punjab, Pakistan, described the prevalence of *Hyalomma* species as 12%. In this study the prevalence of *Boophilus species*, *Haemaphyslis species* and *Rhipecephalus species* were 8.1%, 5% and 3.1%, respectively. This study is not congruent with our results as *Amblyomma species* were not reported in this study and prevalence rate of

Hyalomma species is significantly low as compared to our study. In lower Punjab (Muzaffargarh and Layyah districts) *H. anatolicum* at 41% was highly prevalent tick species in cattle population followed by *R. sanguineus* at 25.5%, while in buffalo population *H. anatolicum* was at 36.3%, *R. sanguineus* was at 29.6% in this study (Sajid *et al.,* 2008). *Hyalomma species* prevalence in this study is higher as compared to other tick species showing similar results to our study. Another tick infestation study was reported by Manan *et al.,* 2007 in Peshawar region situated in the Frontier province (presently known as KPK) of Pakistan, showed highest prevalence of *Boophilus species* at 46% followed by *Hyalomma species* at 31.25%, *Rhipicephalus species* at 17.93% and *Amblyomma species* at 4.61%. Similar study was performed by Rehman *et al.,* 2004 in Rawalpindi and Islamabad areas which are situated in upper Punjab of Pakistan, showing *Haemaphysalis species* prevalence as 74% and *Hyalomma species* prevalence as 26%. *Boophilus* species, *Amblyomma* species and *Rhipicephalus species* were not reported in Rawalpindi and Islamabad areas.

Table-14 Comparison of different tick genera distribution in cattle and buffaloes from 2001 to 2010

Genus	Name of Author with %prevalence	District of study	species
Hyalomma	Rehman *et al.*,2004 26%	Rawalpindi and Islamabad	Cattle, buffaloes, goat and sheep
	Manan *et al.*, 2007 31.25 %	Peshawar	Cattle, buffaloes, sheep, goat and donkey
	Sajid *et al.*, 2008 41%,36.3% and 42.2%	Layyah and Muzaffargarh	Cattle, buffalo and sheep respectively
	Durrani and Shakoori,2009 12%	Rawalpindi, Lahore and Multan	Cattle
	Atif *et al.*,2012 23%	Sargodha, Khushab and Rawalpindi	Cattle
Haemaphysalis	Manan *et al.*, 2007 46.1%	Peshawar	Cattle, buffaloes, sheep, goat and donkey
	Durrani, and Shakoori, 2009 8.1%	Rawalpindi, Lahore and Multan	Cattle
	Atif *et al.*,2012 1.62%	Sargodha, Khushab and Rawalpindi	Cattle
Amblyomma	Manan *et al.*, 2007 4.61%	Peshawar	Cattle, buffaloes, sheep, goat and donkey
Rhipecephalus	Sajid *et al.*, 2008 25.5%, 29.6 and 37.6	Layyah and Muzaffargarh	(Cattle, buffalo and sheep respectively
	Manan *et al.*, 2007 17.93%,	Peshawar	Cattle, buffaloes, sheep, goat and donkey
	Durrani and shakoori, 2009 3.1%,	Rawalpindi, Lahore and Multan	Cattle
	Atif et al.,2012 23%	Sargodha, Khushab and Rawalpindi	Cattle

Our study demonstrated the occurrence of adult female ticks to be significantly higher **as** compared to adult male ticks. Similar results were described by Sayin *et al*. (2003) showing higher distribution of female ticks as compared to male ticks. In *Hyalomma* species, prevalence of female tick population was higher than male tick population as described by Flach *et al.* 1993 and Aktas *et al.* 2004. Ghosh *et al.,* 2007 also reported similar results in a study describing female population as 77% and 72% in *Boophilus species* and *Haemaphysalis species*, respectively in one area of study in Bangladesh. Khan *et al*. Salivary gland of adult female Hyalomma ticks have more type III acini as compared to male describing their more disease transmission potential of female *Hyalomma* tick as theileria parasites are only found in Type III acini (Young *et al.,* 1983). Moreover, histamine binding proteins which counteract pain/response of host to tick attachment are two in female tick as compared to male ticks (Brossard and Wikel, 2004).

Similar result of tick infestation was reported by Sajid *et al.,* (2008) describing 75.1% cattle infested with ticks as compared to buffaloes with 40% infestation rate. These findings are in line with our study in which we estimated tick infestation rate in cattle and buffalo population. In cattle and buffaloes tick infestation was also observed by Rehman *et al.,* (2004) and Manan *et al.,* (2007) describing double magnitude of ticks in cattle as compared to buffaloes. In tick infestation level study high infestation level (tick number above 50) was higher at 59% in cattle as compared to buffalo high at 18%. Moderately infestation level (tick umber 25-50) was higher in buffaloes at 38% as compared to cattle at 23%. Again there was a significant difference between buffalo and. In the category of

low infestation level (1-25), buffaloes has 44% as compared to cattle population at 18%. High tick infestation rate and high tick level infestation in cattle may due to the reason that majority of cattle in the area of study were cross bred which are more vulnerable to tick infestation (Fesharki, 1988). Tick infestation level study in cattle and buffalo population is not reported previous in the literature in these areas.

During study it was observed that animals tethered under trees have tremendously low level of tick infestation than those animals which are kept in sheds. This was due to tick eating habits of predator birds perching on the trees. In these areas small farm holders usually keep poultry in their houses as poultry can not only pick ticks from the body of animals but also from corner and crevices. Similar approach of tick biological control is being used in some parts of America by rearing guinea fowl as Lyme disease tick eater (reported in unpublished data). In our study, it was also observed that farmers spread sand under and around their animals which is a type of physical control method of ticks.

2. Detection of T. annulata in tick species by PCR

The results of PCR Analysis showed the detection of *T. Annulata* in *H. annatolicum* and *H. dromedari* but the protozoa was not detected in *H. marginatum. Rhipicephalus (Boophilus) annulatus and A.variegatum.* Molecular detection survey of *T. Annulata* by PCR from hard ticks has not been yet performed in Pakistan.

In conclusion, the tick population in cattle and buffaloes are higher particularly *Hyalomma* species which are member of *Ixodidae* (hard ticks) and are potential vector for the tropical Theileriosis in bovine and other wild animals not only in Pakistan and but also in other regions like Europe, Africa, South Asia and Middle East (Dumanli *et al*., 2005). As compared to previous studies (Table-7), higher prevalence of *Hyalomma species* demands better integrated pest management measures for the control of tropical theileriosis. Secondly, it is the first report that described the prevalence of *T.annulata* in the ticks in Pakistan, which proves that ticks are spreading tick borne diseases in livestock population of the country. Moreover disease transmission potential for other prevalent ticks is also important like *Rhipicephalus (Boophilus)* (bovine babesiosis and anaplasmosis), *Haemaphysalis* (theileriosis and babesiosis in sheep and goats) and *Amblyomma* (Heart water in cattle).

3. Tick susceptibility due to differences in breed, sex and age of host

Holstein cows in mid-lactation period were artificially infested with tick larvae to estimate host resistance against ticks. It was estimated that tick resistance in this breed could be effective with out decreasing milk production (Jonson *et al.,* 2000). Prevalence of ticks was significantly higher in female cattle at 59.7% as compared to male at 35.83% It was also estimated that tick infestation was more in young cattle at 46.28% as compared to adult at 27.8% (Kabir *et al.*, 2011). During feeding, ticks inject its saliva that

contain a variety of enzymes, toxins, enzyme inhibitors, anticoagulants and vasoacfive substances that facilitate effective feeding . These pharmacopia in tick saliva produce immune response in host vertebrates and make the animal resistance tick ifestation (Abdel Wahab *et al.*, 2000).

Non-chemical control method like immunization of susceptible animal species may have an additive effect as artificial active acquired immunity could be a better alternate of therapeutics application. Immunization results into reducing the number of engorged ticks, weight and capacity of egg laying (Willadson *et al.*, 1996), thus enhancing cattle protection against blood sucking, immune-suppression, skin damage and tick borne diseases (Andreoti *et al.*, 2002). Immunoglobins produced after immunization is major component of host serum as they can cross the tick gut and react with tissues in hemocoele (Brossard and Wikel, 2004). In this way tick vaccine may affect the transmission of tick borne diseases by decreasing vector capability of ticks (de la Fuente and Kocan, 2006).

4. Effects on tick reproductive efficiency by immunization

Developmental and reproductive performances of adult *H.anatolicum* ticks were observed in cross-bred calves by immunizing whole homogenate *Hyalomma* vaccine. The vaccine mitigated egg mass, engorgement weight and reproductive index in vaccinated animals because this vaccine contains exposed as well as concealed antigen.

Salivary contents as exposed antigens induce local hypersensitivity reactions which results into restriction of blood flow to the tick. Intestinal contents as concealed antigens provided local damage to gut resulting into gut perforation due to cell mediated lyses. In immunized animals, rejected adult female ticks were white to pale yellow in color due to the lack of red blood cells intake in their meal. This may suggest the decrease in transmission of tick borne diseases due to reduced pick up of infected RBC'S.

Immunization of animals with tick vaccine although interfere the tick life cycles and their reproductive capacity but there are no observations of high tick mortality (Rodriguez *et al.,* 1995 and Willadson, 1989). In our study percentage rejection of adult female *H.anatolicum* tick was 68.8567±1.8687 which is significant higher than controlled group. Das *et al.* (2000) purified extract of unfed larvae of *H.a.anatilucm* by immunoaffinity chromatography and used for immunization of cross-bred calves. In their study rejection percentage was 75.0±3.42 and 70.6±10.2, respectively, which is in agreement with than that of our study. Slightly higher rejection percentage might be due to use of purified antigen as a source of vaccine.

In engorgement period there was non-significant different between vaccinated and controlled group. In vitro conditions egg laying mass in immunized group was 151.67±4.40 which was significantly higher as compared to controlled group. Similarly engorged weight in immunized group was significantly high as compared to control group. This indicates the disruption of feeding process of ticks due to immune response

induced by the vaccine in cross-bred calves. Similar parameters were used by Sangwan *et al.* (1998) in their study, reproductive performance of immunized group was poor as compared to control group but statistically there was no significant difference in these parameters except reproductive index. Our study provides overall 69% protection of adult ticks by immunization of whole homogenate tick vaccine in cross-bred calves, in comparison to 55.8% protection provided by unpurified antigen of salivary gland (Saran, 1996). This variation is inline with our another study where immune response to whole homogenate vaccine in rabbits was better as compared to vaccine prepared from salivary gland extract.

Similar criteria for assessing the efficacy of tick vaccine was used (Rechav and Dauth, 1987; Varma *et al.,* 1990; Opdebeeck *et al.,* 1988a and Willadson *et al.,* 1996) and their results are in line with our study. We prepared whole homogenate vaccine from adult female ticks and challenge infestation was later provided with adult female ticks of same species. Mulenga *et al.* (2000) characterized a 29 kDa salivary gland associated protein from *Haemaphysalis longicornis* which led to significant reduction in adult female engorgement weight. Howerver, Varma *et al.,* 1990; Ghosh *et al.;* 1998 and Ghosh and Khan, 1999 have shown that rabbits and cattle were protected significantly against adults of *Rhipecephalus appendiculatus* and *Hylomma a. anatolicum* following immunizing with the immature stage. In future study, immune response of vaccine prepared from local strain of adult tick might be monitored in the immature stages of same species.

Our rationale of applying ELISA on immunized and non-immunized group was to co-relate the immune response produced by whole homogenate vaccine with feeding and reproductive performance of the ticks after immunization. Up to 15 days of post vaccination, there was no significant difference between antibody titer of immunized and non-immunized group due to slow rise in antibody titer in serum. The result of present study shows that tick infestation can be controlled in the area by using immunoprophylactic measures. After boosting, rapid rise in antibody titer was detected; this is a characteristic of memory response. On day 45, the rise in antibody titer in cross-bred calves of control group was due to challenge infestation of ticks that was equally provided in immunized and control group. A significant difference in ELISA titer value was observed between immunized and non-immunized group.

It has been reported that a vaccine against ticks would progressively control ticks and tick borne diseases in successive generations rather controlling tick population in single generation (Patarroyo *et al.*, 2002). The principle of such immunization is uptake of blood meal, tissue fluid or wound exudates which inevitably carry antibodies, complements and other components of host immune system (Tizard, 2000). There are three ways of complement activation (Tortora, 2007), the alternative path-way of complement activation is involved in the host-tick interaction (Wikel, 1979). Acquired resistance to ticks may be based on circulating anti-tick IgM and IgG antibodies as well as Th1-cell-mediated delayed type hypersensitivity reactions, modulated in part by IL-2

and IFN-g (Wikel and Bergman, 1997). Control of hard ticks has been difficult as they have fewer enemies (de la Fuente and Kokan, 2006) and as a whole arthropod parasites are difficult to immunize against because being ectoparasites they feed intermittently from different host, (Opdebeeck, 1994).

5. Vaccination studies

5.1 Different tick organ tissues as candidate antigen

Tick GARD (*Escherichia coli* expressed BM 86 vaccine) and GAVAC (*Pichia pastoris* expressed BM 86 vaccine) have been developed against infestation of *B. microplus* and these vaccines may have mitigated use of acaricides (Anderotti *et al*., 2002). Presence of only one immunogen in recombinant tick vaccine seems to be a plausible reason of less attraction of the vaccine to be used against tick infestation (Mulenga *et al*., 2000). However, different attempts have been made to immunize animals against the ticks by using crude extract (Sangwan *et al*., 1998). Crud immunogenic extracts from different stages of *H. a. anatolicum* have been found protective against challenge infestatation (Manohar and Banerjee, 1992; Sangwan *et al.*, 1998 and Ghosh *et al.*, 1998) but information about the potency or efficacy of such crude extract is scanty.

Different efforts have been made previously to immunize rabbits against *H. anatolicum* using crude extract antigen prepared from different tissues of the tick. Histological structure of hard ticks is mainly composed of chitinous coverings, legs, mouth parts, and internal organs like salivary tissues and intestinal epithelium lining the body cavity. In

our study, organ-based vaccines were prepared from extracts of salivary glands (SGE), intestinal (ITI) tissue and whole tick homogenate (WTH) of *Hyalomma* ticks. Salivary gland is a paired organ consisting of grapelike clusters of acini extending from the level of the peritremes along the sides to the gnathosoma, where the paired main ducts open into the salivarium, which opens dorsally into the buccal canal. Salivary glands are essential for the biological success of ticks and induce number of pharmacopeia during feeding on mammalian host. These pharmacological agents are anticoagulants, prostaglandin, vasodilators, apyrase, antiinflammatory agents, anti-histamines etc. (Bowman and Sauer, 2004). The importance of multi-functionary salivary gland to tick survival and vector competency makes the gland a potential target for intervention (Bowman and Sauer, 2004) and an attractive immunogenic source (exposed antigen) for vaccine development against TBD (Valenzuela, 2004). Tick salivary pharmacopeia also potentiates infection with several infectious agents like tick-borne encephalitis virus, *Borellia burgdorferi sensu stricto* and *Franciscella tularensis* through saliva-activated transmission (SAT) (Nuttal and Labuda, 2004). Tick saliva often contains high concentration of prostaglandins (Bowman *et al.,* 1996) which are vasodilators (Riebero, 1995a) and inhibitors of platelet aggregation.

Intestinal tissue acting as concealed antigen can also be a source of tick antigen as antibody engorged from successfully immunized host blood forms antigen-antibody complex with the help of complements and causes damage to tick intestine. Muslin cloth filtrate of whole tick homogenate contained mainly extract of intestinal epithelium and

salivary gland, so it has both the qualities of exposed antigen as well as concealed antigen. The immune response of vaccines prepared from each of SGE, ITE and WTE was monitored and compared by AGPT in rabbits. The antibody response of rabbits to WTH protein was better than that of vaccine prepared from isolated salivary gland or intestinal tissue homogenate because WTH contains salivary gland components (exposed antigen) as well as intestinal components (concealed antigen). The reason is that immunity induced by natural antigen (exposed) is believed to be inferior as compared to concealed antigen due to reduced antigenicity of natural antigen (Tellam *et al.,* 1992). One drawback of non-salivary gland vaccine is that this

may not absolutely prevent feeding of ticks resulting into transmission of tick borne diseases and hide damage (Ribero, 1987).Valenzuela *et al.,* 2004 purified an anti-complement protein from *I. scapularis* saliva which appears to inhibit C3b binding and accelerate upcoupling of factor Bb from alternative pathway C3 convertase.

5.2 Cross-reactivity study

A cross-reactivity study amongst different prevalent genera was conducted. In conclusion, whole tick homogenate (WTH) of *Hyalomma species* shows cross reactivity with WTH of *Boophilus* and *Amblyomma species*. The results are in agreement to Kawather *et al.,* 2006; de Vos *et al*. 2001 and de la Fuente, 2006 who reported evidence of cross reactivity between different hard tick species. They noted that Tick GARD which

is *B. microplus* derived recombinant Bm86 vaccine is effective against *Hyalomma, Rhipecephalus* and *Amblomma species* of hard ticks. Bm86 is a membrane-bound glycoprotein of 89kDa on the microvilli of tick gut digest cells (Willadsen *et al*., 1989). Bm86 has been expressed in *E. coli*, insect cells using a baculovirus vector (Rand *et al*., 1989) and re-cloned from an Argentine isolate of *B. microplus* and designated BM95 (Garcia Garcia *et al*., 2000). The amino acid sequence of a Bm86 homologue found in *H. a. anatolicum* demonstrated the conservation of this molecule in this species. Gavac vaccine containing the *B.microplus* gut antigen Bm86 showed high efficacy against *B.annulatus* tick infestation in Mexico and Iran (Fragoso *et al.*, 1998). Vaccine made against one tick species can be used against other tick species if cross reactivity occurs between their antigens. Moreover, existing tick vaccines could be improved by the inclusion of additional tick antigens in case of existence of cross-protection.

Sangwan *et al.* (1998) performed a study in which they derived antigens from partially engorged nymph of *H. anatolicum anatolicum* and used in immunization of cross-bred cattle against adult *H.a.anatolicum*. This antigen provided no cross protection against the antigen from adult stage and the results differ with our observations. Rechav *et al.* (1989) artificially immunized guinea pigs and rabbits with larvae of any one of five species of ticks, *Rhipicephalus appendiculatus* Neumann, *Rhipicephalus evertsi evertsi* Neumann, *Amblyomma hebrauem* Koch, *Amblyomma variegatum* Fabricius and *Ixodes ricinus* L. Resistanc was conferred in the animals when exposed to subsequent infestations with the

same tick species. Resistance to infestations was species specific and protection was not observed against other tick species.

Similarly, anti-sera raised against 64 truncated recombinant proteins (TRPs), a secreted cement protein from salivary gland of *Rhipicephalus appendiculatus* showed cross reactivity with crude extract proteins of *Ixodes recinus* and *Rhipicephalus sanguineus* (Trimnel *et al.,* 2005). Two larval glycoproteins 34 kDa and 29 kDA were isolated from larvae of *Hyalomma anatolicum* and *Boophilus microplus* respectively. Cross reactivity and synergistic effects were observed between these two glycoprotein (Ghosh *et al.,* 2005). In another study, truncated constructs of 64P (64TRP'S), a secreted cement protein from salivary glands of the tick *Rhipicephalus appendiculatus* also provided cross reactivity against *R. sanguineus* and *Ixodes ricinus*. Apparently, this protection was provided by making target of antigens in the mid-gut and salivary glands (Trimnell, 2005). Subolesin, a highly conserved protein discovered in *I.scapularis* by expression library immunization was protective against all developmental stages of *I. scarpularis* (Almazan *et al.,* 2003).

These results suggest the feasibility of controlling infestation by multi tick species using protective antigen from one source tick species. It can be also suggested that tick vaccine in future may be formulated by inclusion of multiple antigens which will have broader possible applications.

5.3 Dose of antigen

A study was conducted to optimize the concentration of tick immunogen per dose in vaccine that would provide detectable antibodies in the immunized buffalo calves. For this purpose CFT was performed by using guinea pig as complement source because it contains greater concentration of all complements. Complement has the ability to cause the hemolysis of sensitized sheep RBC'S and combines with antigen-antibody complex causing its fixation. The immunized buffalo calves showed detectable level of anti-WTH complement fixing antibodies in response to the oil based WTH vaccine containing more than 7.5 mg protein/dose.

The antibody titer started increasing on day 15 post priming, reached to peak level on day 45 post-priming and declined thereafter. It was further noted that antibody response of buffalo calves to vaccine containing 7.5 mg protein was not significantly higher than that of the vaccine dose containing 10.0 mg protein *($P<0.05$)*. These results are in agreement with that of Andreotti *et al.* (2002), who observed high level of anti- *Boophilus microplus* Trypsin Inhibitors (BmT1s) antibodies in *Bos indicus* Nelore calves on 40 days post priming with subcutaneous injection of the vaccine (0.1 mg protein/ dose). In their study high level of ELISA antibodies to low amount of immunogen could be due to highly sensitive technique for antibody detection. However, Jackson and Opdebeeck (1990) observed significant increase in total immunoglobulin in serum of cattle immunized with small amounts of mid-gut membrane antigens (500ug) in Quil A (Pipano *et al.*, 2003).

Opdebeeck *et al.,* 1988a protected cattle with 2mg and then 500ug of crude midgut antigen from *B.microplus*. In our study SGE, ITE and whole homogenate vaccines with 2.5mg dose provided detectable immune response in rabbits. In guinea pigs, 200ng of partially purified salivary gland antigen from *A. americanum* was inoculated. This induced a low level of protection (23.4 %) due to lower dose of antigen (Brown and Askenase, 1986).

We inoculated whole homogenate vaccine with 7.5mg concentration in single dose, but vaccine inoculated in multiple and small doses have been shown better immune response as compared to large and single dose (Murray *et al.,* 1979 and Oldham, 1983). In future study, 7.5mg immunogen may be inoculated in crossbred calves in multiple doses to monitor immune response and effect of tick rejection. Vaccine efficacy study was conducted on limited experimental scale and Crossbred animals. It is suggested that field trials in large population may be conducted to increase the strength and precision of results. Moreover, various bovine breeds should be included in the field trials to confirm the potency and efficacy results. The result of present study shows that tick infestation can be controlled in the area by using immunoprophylactic measures. The future of research on tick vaccine development is exciting and lot of work is still to be done in the field of identification of candidate vaccine antigens and exploiting the recombinant technology in the synthesis of these antigens. Development of vaccine that target both ticks and pathogen transmission would be the required goal of these research projects.

Invaluable information from tick genomics may be exploited for the development of tick vaccine. Immunization of these tick vaccines to animal population may also restrict transmission of tick borne pathogens to human population at risk. The results of our study showed that the vaccine prepared in this way may be contaminated with *T. Annulata* –the causative agent of theileriosis. It is therefore recommended that buparvaquone be administered to animals before immunization of this vaccine.

6. Growth of tick intestinal cell as monolayer

The efforts were made to grow midgut cells in insect culture media after isolation the cells from *Hyalomma* ticks. The gut of arthropods is composed of foregut, midgut and hindgut divisions. The midgut may be further divided into anterior and posterior regions and is the site of action for blood meal digestion and absorption. The majority of cells in midgut are digestive in nature and there are four different types of these digestive cell which have secretory vesicles (Marquardt *et al.,* 2005). Midgut is the region where antigen-antibody complex is formed with the help of complement which leads to perforation of midgut. The isolated epithelium cells got confluence rate up to 40% in seven days and then started distorted after each effort due to some contamination. There are different sources of contamination for the tick cell culture which are extrinsic as well extrinsic. The purpose of this experiment was to grow midgut cell and then use these cells as a source of antigen for vaccine development. The growth rate of these cells in our study was no optimal so the media was not split to get more cells.

CHAPTER 7

SUMMARY

A study to investigate prevalence of different genera of hard ticks was carried out in three districts of the Punjab province, Pakistan (Faisalabad, Jhang and Khanewal). Overall prevalence of *Hyalomma species* is the highest at 61% as compared to other genera of hard ticks. In sex-wise distribution, it was found that female *Hyalomma species* were the highest at 85% followed by *Amblyomma species* at 81%, while *Rhipicephalus (Boophilus) species* and *Haemaphysalis species* were at 77%. Infestation rate in cattle at 70% was higher as compared to buffaloes at 34%. In tick infestation level study, high infestation level in cattle at 59% was higher as compared to that of buffalo population at 18%. In cattle population, PCR results showed the prevalence of *T. annulata* in *H. anatolicum* and *H.dromedari* ticks as 50% and 40% respectively. No theilerial organism was detected in *H.marginatum, Rhipicephalus (Boophilus) annulatus* and *Amblyomma variegatum* ticks.

Three different types of vaccines were prepared from different organs of ticks i.e., salivary gland, intestine or whole ticks of the same species of *Hyalomma* and they were injected to rabbits. It was found that vaccine prepared by grinding whole tick produced the higher level of antibody as compared to two other vaccines. Each of the whole tick homogenate vaccine prepared from either of the species of *Hyalomma, Rhipicephalus* or *Amblyomma* and injected to rabbits. These vaccines produced antibody as well and cross reacted with each other showing each of the hard ticks were antigenically similar.

Efforts were made to prepare oil based whole *Hyalomma* tick vaccine with three different antigen concentration 5.0 mg, 7.5mg and 10.0 mg and evaluated its potency in buffalo calves. It

was found that the vaccine dose containing 5.0 mg antigen per dose did not produced detectable antibody in buffalo calves while the vaccine containing 7.5mg or more antigen produced detectable antibody. Moreover, we concluded that montanide based hard tick homogenate vaccine with more than 7.5mg protein per dose is effective in producing antibodies against tick infestation in the dairy animals. The antibody level in vaccinated buffaloes as well as in vaccinated rabbits reached to peak level on day 45 post vaccination and started declining thereafter.

Capacity of vaccine in controlling tick infestation was assessed in 12 cross-bred calves. It was found that rejection percentage in immunized group was higher as compared to control group. There was no difference of engorgement period between immunized and control group. Reproductive index in immunized group was lower as compared to control group.

The efforts were made to grow midgut cells insect culture media after isolation them from *Hyalomma* ticks. The purpose of this experiment was to grow midgut cell and then use these cells as a source of antigen for vaccine development. The isolated epithelium cells got confluence rate up to 40% in seven days and then started distorted after each effort due to some contamination. There are different sources of contamination for the tick cell culture which are extrinsic as well extrinsic. The growth rate of these cells in our study was not optimal so the media was not splitted to get more cells.

CHAPTER 7

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APPENDICES

Appendix #1 Composition of Phosphate buffer saline (1X)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled water	upto 1000 ml

Appendix #2 Veronal buffer (diluent/ stabilizer in CFT)

$CaCl_2 \cdot 2H_2O$	185.4 mg
$MgCl_2 \cdot 6H_2O$	840 mg
NaCl	42,500 mg
Sodium Barbital	1,870 mg
Barbital	2,870 mg
distilled water up to1000ml	(pH 7.28-7.64)

Appendix #3 General plan for determination of agglutination level of the amboceptor

Reagents	Well No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Phosphat Buffer saline pH:7.2	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl
Amboceptor	50µl	-	-	-	-	-	-	-	-	-	-	-
Quantity of mixture transferred	-	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	-
Quantity of mixture discarded	-	-	-	-	-	-	-	-	-	-	50µl	-l
Quant. of 5% washed sheep RBCs	50µl	50µl	50µl	50µl	50µ	50µl	50µl	50µl	50µl	50µl	50µl	50µl
Dilution of the amboceptor	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	-

Positive= Haemagglutination Negative= Clear button (bead) formation in centre of the wells

Appendix #4 Calcµlation of four hemolytic units

Reagents	**Micro-titer plate wells**											
	1	2	3	4	5	6	7	8	9	10	11	12
P.B.S	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl
*G.P. serum	50µl	-	-	-	-	-	-	-	-	-	-	-
**Quantity of mixture transferred	-	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µL	50µl	-
Quantity of mixture discarded	-	-	-	-	-	-	-	-	-	-	50µl	-
Quantity of 0.2% sensitized sheep RBCs	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl
Dilution of the G.P. serum	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	

PBS= Phosphate buffered saline (pH 7.2) containing calcium and magnesium ions

* = GP = Guinea pig ** = Quant. = Qauntit

Appendix #5 Plane of the complement fixation test

Reagents	Micro-titer Plat Wells									Control well *		
	1	2	3	4	5	6	7	8	9	10[a]	11[b]	12[c]
P.B.S	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl
Serum	50µl	-	-	-	-	-	-	-	-	-	-	-
Quant. of mixture transferred to	-	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	-	-	-
Quant. of mixture discarded	-	-	-	-	-	-	-	-	50µl	-	-	-
Antigen	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	-	-
Quant. of 4 HL of complement	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	-
Quant. of 0.2% washed sheep RBCs	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl	50µl
Dilution of the serum	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	-	-	-

Negative = Lysis of RBCs Posative = No lysis/ sedimentation of RBCs

CFT = Complement fixation test, PBS = Phosphate buffer saline Quant. = Quantity,

4 HL = Four hemolytic units, RBCs = Red blood cells

* Control = Well No. 10,11 and 12 were used as control for complement, antigen and sensitized RBCs.

a = Well 10 for complement control, b = Well 11 for antigen control, c = Well 2 for sensitized RBCs contro

Appendix #6 Coating buffer for ELISA 50mM (pH: 9.6)

Sodium carbonate	(Na_2CO_3)	10.60 gm
Sodium bircarbonate	($NaHCO_3$)	8.4gm
Sodium azide	($NaNO_3$)	0.20gm

Make volume up to 1000ml with distilled water and adjust pH:9.6 with HCl or NaOH.

Appendix #7 Washing buffer for Elisa

Phosphate buffer saline (1x)	1000 ml
Tween 20	0.5ml

Appendix #8 Citrate phosphate buffer (pH: 5)

Citric acid	2.14gm
Na2HPO4. 2H2O	3.54gm
Distilled water	400ml

Appendix #9 Meteorological conditions in three districts of Punjab

District Faisalabad

MONTH	MONTHLY RAIN FALL(MM)	MEAN MONTHLY TEMPERTATUE	MEAN MONTHLY HUMIDITY
Sep-06	86	32	64.5
Oct-06	20	26	63.5
Nov-06	9	19	73.5
Dec-06	46	14	75
Jan-07	1	12	66
Feb-07	37	15	75
Mar-07	34	19	66
Apr-07	0	29	42
May-07	21	32	34
Jun-07	13	34	49
Jµl-07	231	31	64
Aug-07	1	32	62

District Jhang

MONTH	MONTHLY RAIN FALL(MM)	MEAN MONTHLY TEMPERTATUE	MEAN MONTHLY HUMIDITY
Sep-06	2	30	63
Oct-06	7	26	66
Nov-06	11	20	78
Dec-06	29	14	78
Jan-07	-1	12	67
Feb-07	43	15	75
Mar-07	44	19	69
Apr-07	2.8	29	46
May-07	8	32	39
Jun-07	154	33	56
Jµl-07	113	32	66
Aug-07	4	33	66

District Mµltan

MONTH	MONTHLY RAIN FALL(MM)	MEAN MONTHLY TEMPERTATUE	MEAN MONTHLY HUMIDITY
Sep-06	-1	31	56
Oct-06	4	28	59
Nov-06	3	21	67
Dec-06	31	15	70
Jan-07	-1	13	61
Feb-07	88	16	70
Mar-07	36	21	62
Apr-07	0.5	31	36
May-07	0.1	34	34
Jun-07	66	35	48
Jµl-07	25	34	58
Aug-07	7	33	59