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Investigating The Molecular Aspects of Theileria Annulata In Naturally Infected Animals, Alongside A Mention of Tick Distribution In Hyderabad And Karachi

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Abstract: Introduction: The *Theileria annulata* is a haemo protozoan parasite that causes economically significant fatal tick-borne diseases in domesticated and wild animals. **Methodology:** The research focused on identifying *Theileria annulata* infection in both cattle and buffalo through molecular methods. To achieve this, DNA extraction was conducted using whole blood samples, followed by the design of specific primers for *Theileria annulata* and subsequent PCR (Polymerase Chain Reaction) analysis. **Results**: Data were also collected on tick infestation and host-parasite relationship. A total of 2400 Cattle and Buffaloes were evaluated during the study. District-wise detection indicated that the highest rate of infected samples was recorded from Peri-Urban (Cattle=88.33%, Buffalo= 61.94%) and Urban areas (Cattle=65.38%, Buffalo=54.10%) of district Hyderabad, while the lowest rate was recorded from Peri-urban (Cattle=24%, Buffalo=17.9%) and Urban (20.38%, Buffalo=16.66%) areas of district Karachi, Month wise detection of *Theileria annulata* indicated the highest rate in July (Cattle= 74.0%, Buffalo=47.5%) and the lowest rate in December (Cattle=11.7%, Buffalo=18.1%). The prevalence of parasitic infection showed a notable increase ($P<0.05$) in the areas of the Suburban region than in Cityscape areas. **Conclusion:** In the molecular identification of *Theileria annulata*, the infection rate exhibited a notable difference between District Hyderabad and District Karachi, with a higher prevalence in the former and a lower incidence in the latter. Additionally, cattle displayed greater susceptibility to tick infestation in comparison to buffalo.

Keywords: PCR, *Theileria annulata* (Parasite), Cattle and Buffalo (Host), Karachi and Hyderabad.

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Introduction

Theileria annulata causes Theileriosis in livestock due to large health and production losses and therefore it remains a challenge and socio-economic threat to the sustainable livestock sector [1,2]. The closely associated protozoan, *Theileria annulata*, exists as an intraerythrocytic microorganism within vertebrates. Its life cycle involves a series of stages, including the tick's transitional growth acting as a potential carrier, possibly through a virus or plasmid containing genetically modified DNA segments that penetrate the host cell. The contentious nature of the tick's sexual stage has sparked ongoing debate. The complexity of its developmental stages and the relatively minimal presence of bacteria contribute to the persistent challenge of finding a definitive resolution to this issue [3]. *Theileria Parva* and *Theileria annulata* pose significant threats to bovines in humid and sub-humid regions, emphasizing the necessity for an effective

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vaccine against these diseases. Evaluations of immune responses to parasite resistance at various stages involve employing immune markers to identify foreign elements that trigger antibody production within the body. The inoculation of cattle with these substances prompts the activation of minute bodies, each originating from the division of Sporozoites, which develop into individual adults or undergo cellular development from a schizont, parasitizing a red blood cell during the host stage. These processes result in varying degrees of reinforcement against the challenge.While the level of fortification paid is unsatisfactory for vaccination, other issues are at hand, including the selection of substances and their distribution system used.

Ongoing research remains crucial in identifying prospective vaccine options. Progressing our understanding in biology entails delving into the complexities of biological occurrences at a molecular level, centering on DNA, RNA, proteins, and other vital macromolecules that impact genetic data and cellular functions using advanced tools and techniques typically segmented. the manipulation, imaging, analysis, and parasitism of parasites obtained during this research also provide opportunities for the developing and enhancing of immediate vaccines [4]. *Theileria annulata* and *T. Parva* are closely linked to host species that may proliferate in animal diseases. Sequence of hereditary characteristics of organic *T. annulata*. Comparing the *T. Parva* Genome, we acknowledge the contrast between change and strategy. While there's anticipation for gene sequences and resemblances, analyses indicate disparities among gene families and specific gene extensions. (1)Additionally, there exist antibodies reactive to specific antigens and free radicals purportedly diminishing resistance in other organisms within the body. Moreover, certain regions of specific proteins exhibit elevated levels of crucial nitrogen in all organic matter [5].*Theileria annulata* and *T. parva* are associated with protozoan parasites capable of eliminating *T. annulata* lymphoproliferative viruses in cattle. Efforts to classify the T. Parva genome involve comparing it to the Parva genome to comprehend evolutionary processes and markers[17]. Despite the well-preserved gene structure and classification, studies reveal significant differences within the genus's family, highlighting distinctions among specific genera. Various classes of anatomical peptides are known to impede immune recognition regulators and host cell processes, including a multitude of Theileria-specific diverse organic molecules integral to Theileria's structure, particularly within modified proteins. Individuals who have encountered tick bites in southeastern Sweden and presented symptoms such as asthma and influenza, alongside prior Borrelia infection, have undergone medical examinations. Specific reports indicate serological evidence of rickettsial infection among these examined patients[18]. Out of approximately 206 individuals, 20 displayed symptomatic paths, exhibiting additional IgG or distinct IgM antibodies against rickettsiae.

As the deadline rapidly approaches, approximately 1 in 64 cases have been observed. Among the roughly 20 pathways identified, 7 displayed serum antibody development linked to ongoing infection, while 13 patients exhibited signs consistent with prior infections. Among the 19 patients with accessible medical records, 5 showed signs of Borrelia activity[4]. Intriguingly, despite the absence of confirmed skin lesions, all 19 patients recovered safely. Noteworthy is that 5 out of these 19 patients displayed antibodies for all three pathogens associated with Erythema migrans or migrating rashes [20]. Various combinations of symptoms, including serum reactivity, fever, myalgia, headache, and dyspnea, were observed. These findings were contrasted with the examination of an additional 159 individuals infected, often screened for Borrelia or Mycoplasma pneumonia. Among these, 16 patients exhibited a serological response to *Theileria annulata*, with five of them being classified as persistently contagious or exhibiting persistent symptoms. Predominant symptoms included arthritis, fever, cough, and rash. Within a group of 80 asymptomatic blood donors, approximately 1% showed a serological response indicating prior exposure to *Theileria annulata.* This study highlights both singular and concurrent afflictions, emphasizing the need for comprehensive investigations to comprehend the complex medical landscape and establish optimal treatment approaches for affected individuals. [6].

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Signs and Symptoms of Theileriosis

There are lots of sign and symptoms of Theileriosis:

- *Theileria annulata* causes infection in lymphocytes.
- Symptoms encompass anorexia, fever, and swollen lymph nodes leading to lymphadenopathy.
- Less prevalent indications may involve diarrhea and nasal discharge.
- An affliction termed "turning sickness" can arise when parasites within cells obstruct blood vessels in the brain, potentially leading to brain damage, Typically, this disease culminates in fatality [16].

Methodology

Collection of Blood Sample

Between 2018 and 2019, a total of 1,783 blood samples were randomly gathered from both cattle and buffaloes residing in the regions of Hyderabad and Karachi, Pakistan. Blood samples were collected from the jugular vein and subsequently transported to the laboratory named Molecular Parasitology at Sindh Agriculture University, Tandojam. The samples underwent thorough examination and Polymerase Chain Reaction (PCR) analysis. [7].

DNA Extraction From Blood

The DNA extraction process followed a specific protocol using the commercial QIAGEN QIAamp® DNA Kit #51305. Here are the steps:

In a 1.5 ml micro centrifuge tube, 20 µl of Protease solution was introduced.

Following this, 200 µl of the collected blood sample and 200 µl of Buffer AL were combined and thoroughly mixed by vortexing.

The mixture underwent an incubation period at 56ºC for 10 minutes, followed by a quick centrifugation step to eliminate any residual drops from the tube's lid.

Subsequently, 200 µl of ethanol (96-100%) was added, and thoroughly mixed by vortexing, and the tube was briefly centrifuged to remove any remaining droplets from the lid.

Using a pipette, the mixture was transferred into a mini spin column (positioned within a 2 ml collection tube) and centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube contents were discarded.

The mini spin column was then transferred to a new 2 ml collection tube, and 500 µl of Buffer AW2 was applied. This underwent centrifugation at 14000 rpm for 3 minutes, and once more, the flow-through and collection tube contents were discarded.

A fresh mini spin column was placed in a 1.5 ml micro centrifuge tube, and either 200 µl of Buffer AE or distilled water was added. This was left to incubate at room temperature (15-25ºC) for 1 minute before being centrifuged at 8000 rpm for 1 minute to elute the DNA.

Certainly! Here's a Breakdown of the PCR Process:

1. **PCR Components and Volumes:** The PCR process utilized components and volumes as outlined in Table 1. The sample tubes containing these components were loaded into a Thermal Cycler (Applied Bio Systems, USA). The program was pre-set, and the operation commenced upon closing the machine's lid.

2. **PCR Cycling Steps:**
   - **DNA Denaturation:** The DNA underwent denaturation at 94ºC for 5 minutes.
   - **Annealing:** The annealing step occurred at 55ºC for 1 minute, allowing primers to bind to the DNA strands.

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- **Extension:** DNA polymerase elongated the primers, producing two complementary DNA copies from a single DNA template at 72ºC for 1 minute. Subsequently, the cycle resumed starting from the initial denaturation phase at 94ºC.

3. **PCR Product Analysis:** Subsequently, the PCR product obtained from these cycles underwent electrophoresis for further analysis or characterization. [13].

Table 1: PCR Master Mix composition

Components	Volume
Master mix	15 µl
Theileria annulata Primer (F)	4µl
Theileria annulata Primer (R)	4 µl
DNA extract	2 µl
Distilled water	5µl
Total	**30 µl**

These components were combined in the specified volumes to create a total PCR reaction volume of 30 µl.

Procedure for Primer dilution and DNA addition:

1. **Primer Dilution:**

Firstly, all primers were diluted using 20 µl of TE Buffer.
For the Primer (F), 8 µl was combined with 25 µl of master mix in a small tube from the Neptune company.
Similarly, for Primer (R), an 8 µl portion was mixed with 25 µl of master mix in a separate small tube also from the Neptune company.

2. **DNA Extraction Process:**

- 2 µl of DNA extract was separately added into the tubes containing Primer (F) and Primer (R), respectively.

This process ensured the incorporation of the primers into the master mix and the subsequent addition of DNA extract to the respective tubes containing the primers.

Table-2 Certainly! Here's the information structured for Primers used:

Primers	Nucleotides	Species	References
T.annulata-F	CACCTTCGACAAGAAAGAAGTCGG	Theileria	Designed in Mather lab, USA
T.annulata-R	TGAGAAGACGATGAGTACTGAGGC	Theileria	Designed in Mather lab, USA

This table outlines the primers used in the PCR process, including the primer names, nucleotide sequences, associated species, and the lab where they were designed.

This is A Detailed Protocol For Preparing a 1% Agarose Gel For Electrophoresis:
Measured out 0.5g of Agarose and placed it in a 250 mL conical flask. Added 50mL of 0.5x TAE buffer and swirled the mixture to ensure thorough mixing.
The mixture was microwaved for about 1 minute to dissolve the Agarose.
Allowed the heated mixture to cool on the bench for 5 minutes until it reached approximately 60°C.
Added 2µL of ethidium bromide (10mg/mL) and swirled the solution for thorough integration.
The cooling step was crucial to reduce the production of ethidium bromide vapor. A solution containing 10mg/mL ethidium bromide could be prepared from tablets, stored at 4°C in a dark area, and labeled as hazardous for safe handling.

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Carefully poured the gel into the tank, using a disposable tip to remove any bubbles and ensuring proper positioning of the comb.
Let the gel solidify for a minimum of 30 minutes.
Added 0.5x TAE buffer into the gel tank, ensuring the gel was submerged to a depth of 2–5mm for electrophoresis.

It is a standard procedure for loading and running samples on an agarose gel for DNA analysis:

1. Sample Loading:
The DNA ladder (from Fermentas EU) was placed in the initial well as a reference for determining sample sizes.
Subsequently, 4 µl of each sample was loaded into the consecutive wells.
The electrophoresis unit was then configured to run at 80 volts and 100 amperes for 30-45 minutes to facilitate the migration of the samples through the gel.

2. Gel Documentation
Post-electrophoresis, the gel was extracted and positioned in a gel documentation system (Cleaver Scientific, Ltd, UK).The bands within the samples were observed and their sizes were assessed by comparing them against the bands of the DNA ladder utilized as a benchmark.

Results
This study aimed to assess the occurrence of *Theileria* species among cattle and buffaloes in the P.U and urban areas of Hyderabad, Sindh, Pakistan. Throughout the investigation, certain animals displayed both tick infestation and clinical symptoms associated with Theileriosis. This suggests a potential correlation between the presence of ticks and the manifestation of Theileria-related signs in these animals. Ranking of predilection, the overall prevalence of Theileria infection was at District Hyderabad (77.67%) in cattle and (58.19%) in Buffalo. The PCR data regarding *Theileria annulata* detection in the P. U areas of Hyderabad suggest the presence of the highest infection rate in this specific geographical region.
88.33% in cattle and lowest in Buffalo 61.94%, while at Urban areas the infection rate in Cattle was 65.38% and 54.10% in Buffalo. (Table 3 and Figures 1 and 2) Out of these 2400 observed animals the tick-infested bovine samples, over al 1046 (87.16%) cattle were infested at P.U and urban areas, and (870) 72.5% Buffalo were infested (Table 3). The PCR findings for *Theileria annulata* detection in both Peri-urban and urban areas of Karachi indicate an overall infection rate of 22.32% specifically in cattle, and 17.39 % in Buffalo (Table 4, Figure 3 and Figure 4), The data of Month-Wise PCR detection of *Theileria annulata* infection in Cattle and Buffalo indicates the highest rate in July (Cattle=74% and Buffalo=47.5%) and lowest in the month of December (Cattle=11.7% and Buffalo= 18.1%) Table 5, Figure 5 and 6.The result of DNA quantity of the blood Sample of Cattle in the Nanodrop spectrophotometer indicates the 36.7 ng/µl and that of buffalo has 7.3ng/µl (Figure 7 and 8) The Gel electrophoresis of the amplified PCR product of *Theileria annulata indicates* the band at 295 base pairs (Figure 9).
This table represents the observed percentages and percentages of infected samples for cattle and buffalo in both Peri-urban and urban areas of Hyderabad concerning *Theileria annulata* detection using PCR. The prevalence of parasitic infection showed a notable increase($P<0.05$) in the areas of the Suburban region than in Cityscape areas.

Table-3: Here's the structured table based on the obtained data:

Districts	Hyderabad cattle	Hyderabad buffaloes

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	Observed	**Infested (%)**	**At Random blood samples**	**Infected samples (%)**	**Observed**	**Infested Samples (%)**	**At Random blood samples**	**Infected samples (%)**
Hyderabad Peri- Urban	600	540 (90%)	300	265 (88.33%)	600	460 (76.66)	226	140 (61.94%)
Hyderabad Urban	600	506 (84%)	260	170 (65.38%)	600	410 (68.33)	207	112 (54.10)
Total	1200	1046 (87.16)	560	435 (77.67)	1200	870 (72.5%)	433	252 (58.19%)

Table-4: Here's is the structured table based on the obtained data of the regions of Karachi

Districts	**CATTLE**				**BUFFALO**			
	Karachi cattle Observed	**Karachi cattle Infested (%)**	**Karachi blood samples**	**Karachi Infected samples (%)**	**Karachi Observed buffaloes**	**Karachi Infested Samples (%)**	**Karachi blood samples**	**Karachi Infected samples (%)**
Peri-Urban	600	232 (38.6%)	300	72 (24 %)	600	160 (26.66)	134	24 (17.9%)
Urban	600	205 (34.1%)	260	53 (20.38%)	600	102 (17%)	96	16 (16.66)
Total	1200	437 (36.41)	560	125 (22.32)	1200	262 (21.83%)	230	40 (17.39%)

This table represents the observed percentages and percentages of infected samples for cattle and buffalo in both Peri-urban and urban areas of Karachi concerning *Theileria annulata* detection using PCR.

Table- 5: Here's the structured table based on the obtained month-wise PCR detection data for *Theileria annulata* infection in cattle and buffalo from 2018 to 2019

Month	**Cattle**			**Buffalo**		
	Total Animal Observed	**Total Animal Infected**	**Percentage (%)**	**Total Animal Observed**	**Total Animal Infected**	**Percentage (%)**
January	20	6	30	36	7	19.4

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February	18	8	44.4	30	10	33.3
March	24	16	66.6	42	17	40
April	26	14	53.8	46	16	34.7
May	28	18	64.2	45	20	44.4
June	16	7	43.7	34	9	26.4
July	27	20	**74.0**	40	19	**47.5**
August	26	17	65.3	43	17	39.5
September	24	15	62.5	42	18	42.8
October	20	7	35	37	10	27.0
November	26	7	26.9	34	8	23.5
December	17	2	**11.7**	33	6	**18.1**
Total	272	137	50.36	462	157	33.98

Figure-1: This is visual representation of the differences in infection rates in Cattle in the regions P.U and urban areas of District Hyderabad.

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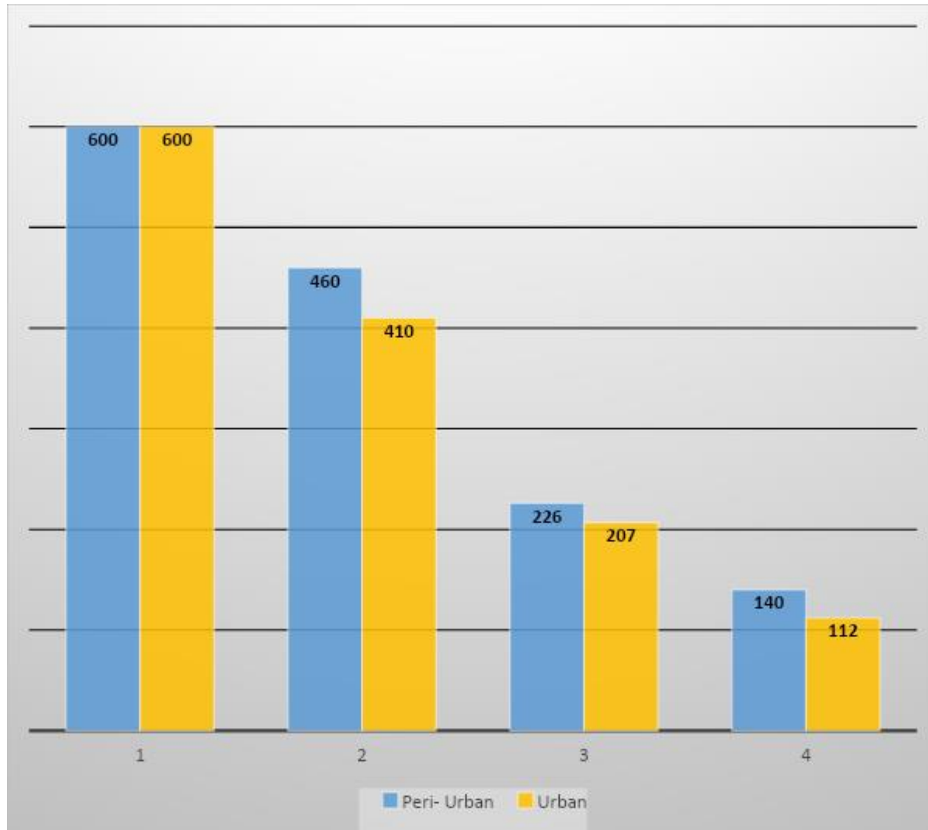

Figure-2: This is visual representation of the differences in infection rates in Buffaloes in the regions Peri-urban and urban areas of District Hyderabad.

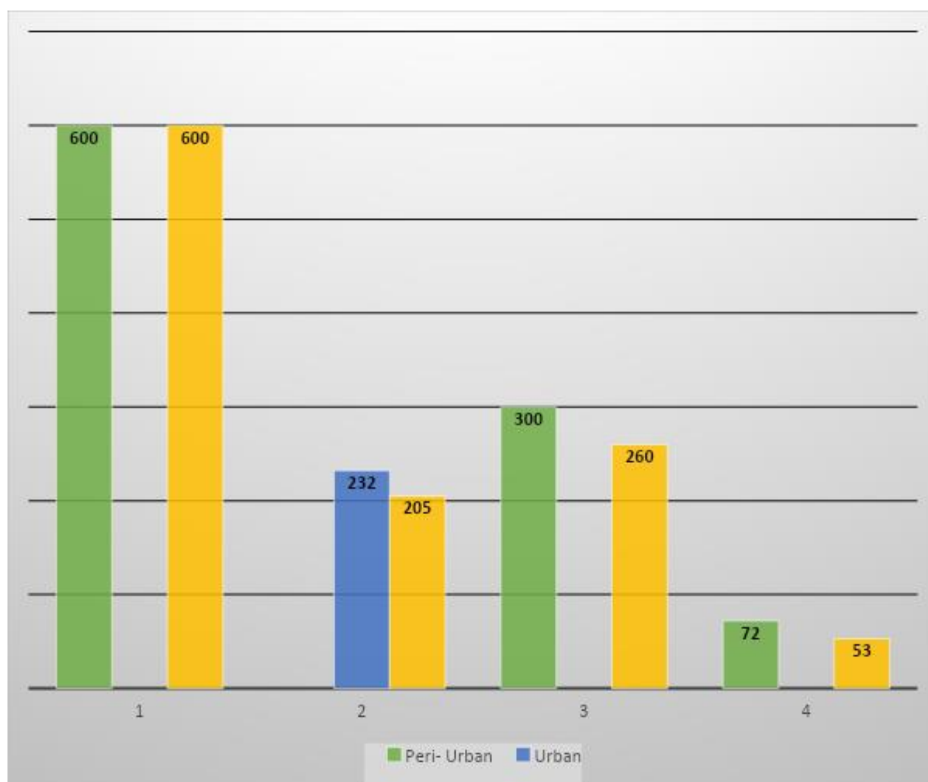

Figure-3: This is visual representation of the differences in infection rates in Cattle in the regions

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of District Karachi.

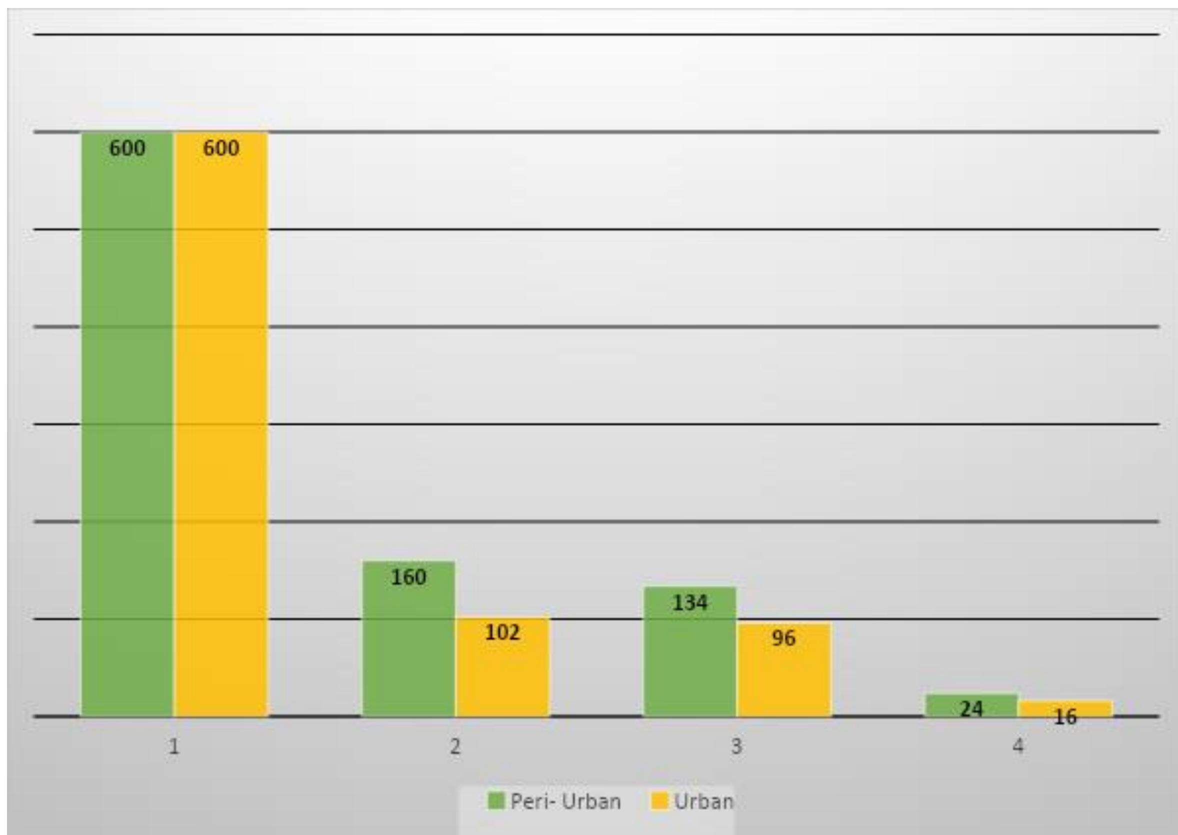

Figure-4: This is visual representation of the differences in infection rates in Buffaloes in the regions of District Karachi.

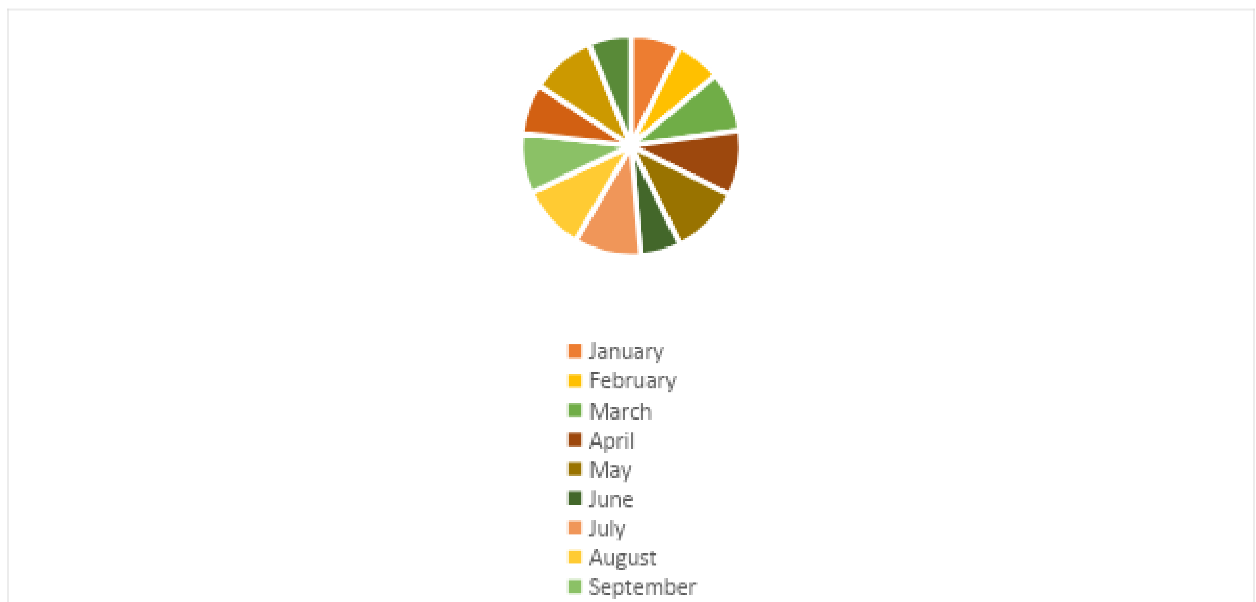

Figure-5: Showing Month wise PCR detection of *Theileria annulata* in Cattle

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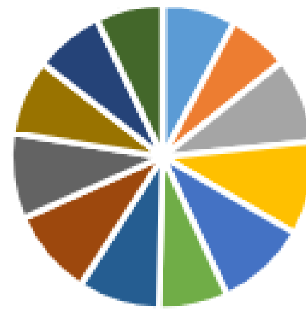

Figure-6: Showing Month wise PCR detection of *Theileria annulata* in Buffalo

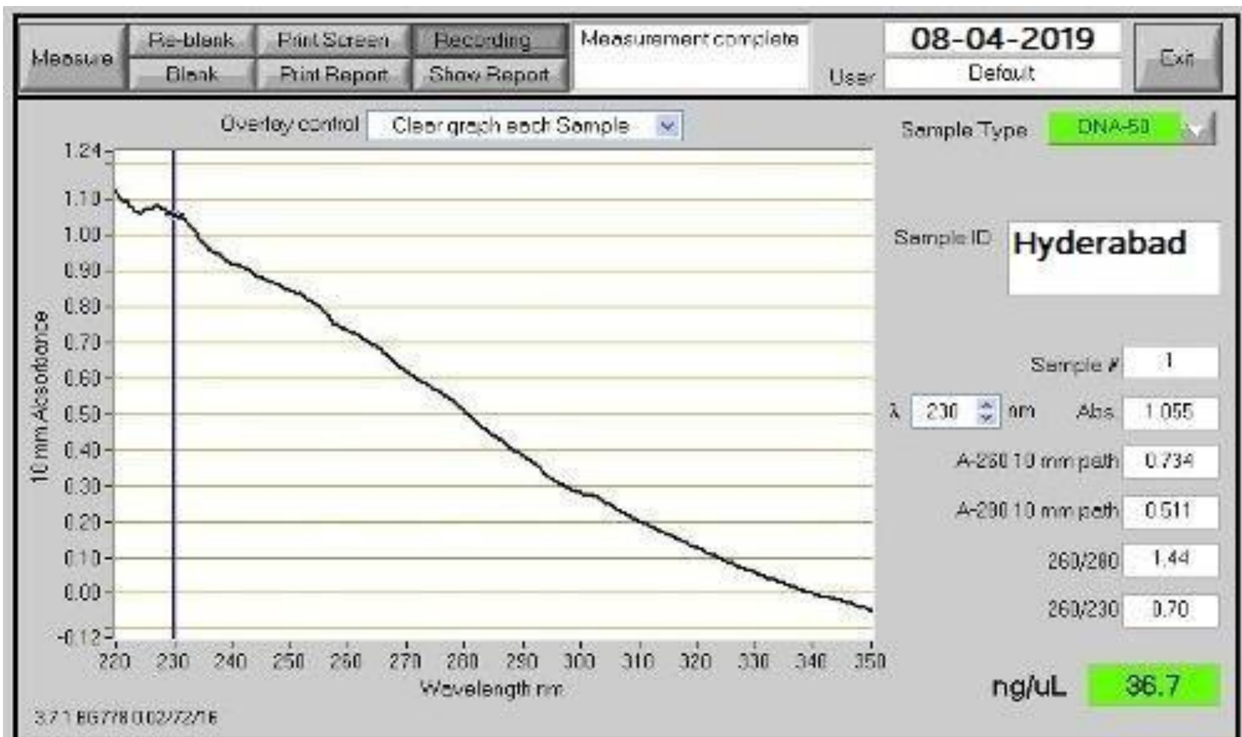

Figure-7: Showing the Quantity of DNA of Blood Sample of Cattle in Nanodrop spectrophotometer

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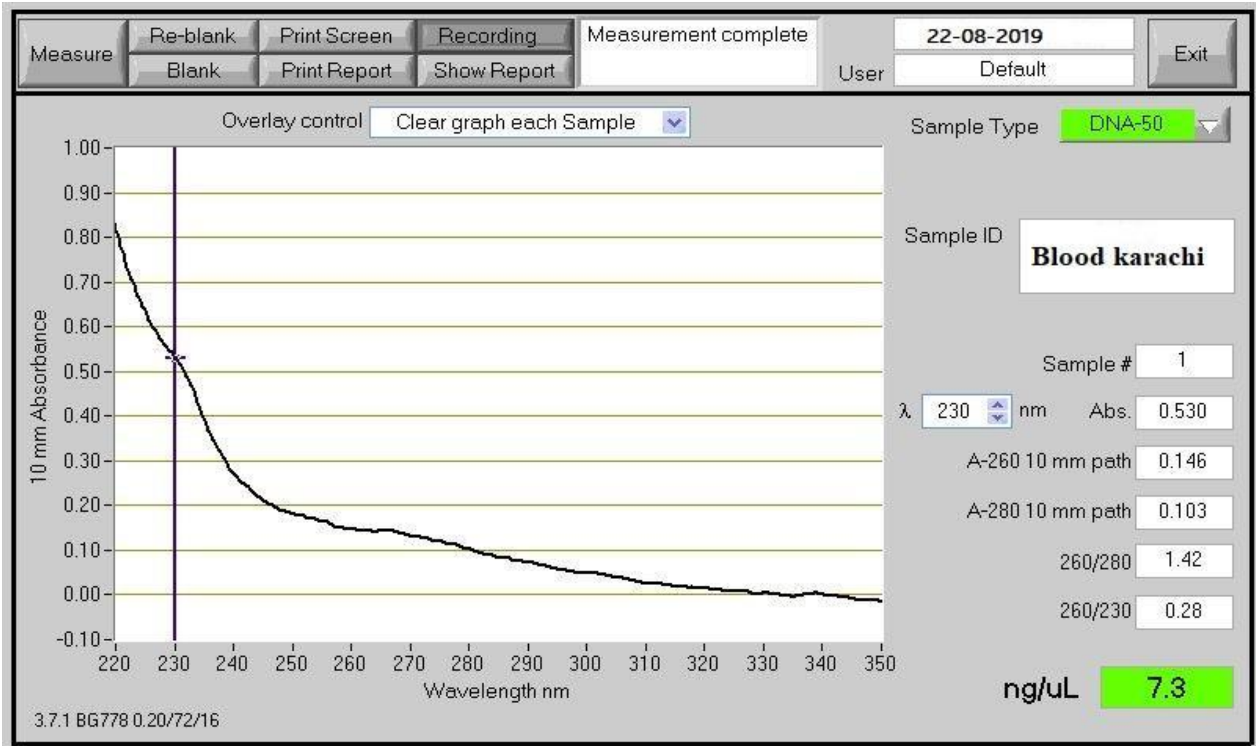

Figure-8: Showing the Quantity of DNA of Blood Sample of Buffalo in Nanodrop spectrophotometer

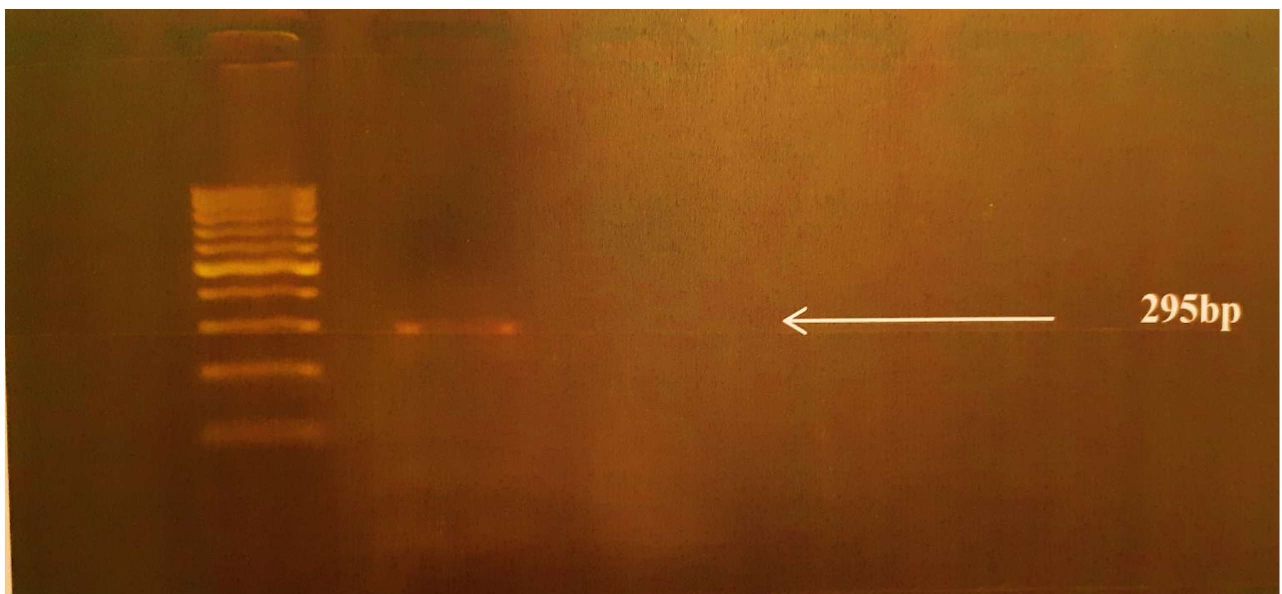

Figure- 9: Showing Gel electrophoresis of amplified PCR product of *Theileria annulata* at 295 bp

DISCUSSION

The purpose of this study is to explore Theileria infection in both cattle and buffalo and its association with tick infestation in different farming regions of Sindh, including Peri-Urban and Urban areas. Similar research findings referenced as [8,9] observed normocytic hypochromic anemia in cattle affected by theileriosis. This condition is believed to stem from persistent blood loss caused by continuous feeding of blood-sucking ticks. In their investigation, they examined various tick glands, notably the submaxillary glands, revealing that 51% of Hyalomma

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anatolicum and 1.3% of *H. anatolicum* were found infected with *T. annulata* sporozoites, indicating their potential role in transmitting the infection.
Additionally, another study referenced as [10] reported the maturity of *T. annulata* in rabbits.
The mentioned study observed that mature *Hyalomma anatolicum* ticks infected with *Theileria annulata* (specifically the Hisser strain) underwent hatching at 36°C or were fed to rabbits. Researchers investigated the tick glands in the oral cavity responsible for secreting saliva, using methyl green pyronin to examine and place them on microscope slides. These samples were then stained with Giemsa's solution for further analysis. Their findings suggested that *Theileria* development in the glands of the oral cavity, which secrete saliva during tick feeding, progressed more rapidly and fully compared to the hatched ticks. furthermore, they show that the fed ticks could be highly adapted to the production of sporozoites for infection. livestock and populations of similar but more stable organisms than a strain. The consequence of the temperature experienced in the background research carried out [11]. which testified to the consequence of the temperature on the diffusion of the racial groups of *Theileria annulata* in *Hyalomma anatolicum*. The study highlighted the significant impact of temperature variations (ranging from 4-40°C) on the shedding process during the tick life cycle and the subsequent spread of Theileria organisms from immature ticks (dryads) to mature adults. Specifically, temperatures above 40°C and below 12°C disrupted the shedding or renewal process.
Additionally, the study assessed the infectivity percentage in the oral glands responsible for saliva secretion, employing a procedure involving methyl green pyronin. It also investigated the effects of heat and humidity across various growth periods of Hyalomma ticks. The findings indicated that optimal levels of heat and humidity notably increased the growth rates of these ticks [12].
Certainly, Theileriosis remains a significant challenge within Pakistan's dairy industry. The prevailing tick species identified include *Hyalomma, Boophilus, Haemaphysalis*, and *Rhipicephalus*. Research indicates a greater occurrence of Theileriosis in cows, notably at 20.83%, in contrast to buffaloes. Among the identified tick genera, Hyalomma displayed a relatively elevated prevalence rate of 6.63%, while Rhipicephalus and Boophilus exhibited lower rates at 1.53%. Interestingly, the Livestock Research Station (LRS) in Islamabad demonstrated an efficient tick management system, reporting no observed tick prevalence. However, rural areas in Islamabad depicted less effective management strategies, manifesting a higher prevalence rate of ticks at 23%[14]. Absolutely, additional research findings underscore the considerable prevalence and impact of tropical theileriosis. Approximately 250 million cattle in various countries including Iran, Turkey, India, and China face the risk of contracting this disease. Theileriosis results in noteworthy economic losses stemming from bovine mortality and reduced productivity among the affected livestock.
There is an urgent requirement to create precise detection tools and efficient medications for treating *Theileria* species. This endeavor seeks to alleviate the economic damages inflicted by this parasitic ailment. In the ongoing research, PCR amplification of the key merozoite surface antigen gene (Tams 1) of the parasite was conducted, potentially as a step toward aiding the creation of more accurate detection techniques and potential remedies for Theileria infections[15].

Conclusions

Our study's findings on the molecular detection of *Theileria annulata* are valuable, showing a higher infection rate in District Hyderabad compared to District Karachi. Additionally, our observation that cattle are more susceptible to tick infestation than buffalo sheds light on the differential vulnerability of these animals to this infection.
Our study serves as a critical indicator of Theileriosis infection rates in animals. Moreover, it emphasizes the importance of employing pesticides to prevent tick infestation in animals, ultimately aiming to mitigate economic losses within the industry. This proactive approach can significantly contribute to averting economic setbacks caused by Theileria annulata infections.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The study on humans was conducted in accordance with the ethical rules of the Helsinki Declaration and Good Clinical Practice.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

None.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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None.

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