Biosight 2023; 04(01): 64-73

Molecular Prevalence of Anaplasma Marginale in Cattle and Identification of Rhipicephalus, Boophilus, Hyalomma, And Amblyomma Ticks from District Mirpurkhas and Hyderabad

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Abstract: Introduction: Anaplasma marginal is a rickettsial organism and is the causative agent in cattle anaplasmosis. This Parasite attacks the red blood cells after infection of sensitive bovine and is transmitted by ticks. **Methodology**: The study was aimed to detect Anaplasma marginale through PCR the prevalence of four genera (Rhipicephalus, Boophilus, Hyalomma, and Amblyomma) of ticks, twelve hundred animals were observed, 560 samples of blood randomly taken from cattle that possesses ticks on their body and these samples were shifted to the Laboratory of Parasitology, Department of Veterinary Parasitology, Sindh Agriculture University, Tandojam. The infected cattle exhibited clinical signs such as Temperature, decrease in appetite, nausea, and chills. The study was conducted over a period of one year from November 2020 to November 2021. **Results**: The results of the study showed a high rate of Anaplasma infection (88.33%) and a prevalence of ticks at Mirpurkhas. Month-wise data reveals that the maximum infection was shown in the month of June and the minimum in the month of November at District Mirpurkhas, while at Hyderabad, the month-wise data indicates the Maximum infection rate in the month of May, and the minimum infection was found in the month of November. **Conclusion**: Ticks collected from two districts of Mirpurkhas and Hyderabad proceeded for identification of Tick's genus, and the mostly ticks which were infested on animals concerned with the Hyalomma genus (26.11%) Amblyomma genus (25.37%), Boophilus genus (24.62%), and Rhipicephalus genus (23.88%).

Keywords: PCR, *Anaplasma marginale* (Parasite), Cattle (Host), Mirpurkhas, Hyderabad.

Received: 21-10-2022
Accepted: 19-12-2022
DOI: 10.46568/bios.v4i1.93

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Introduction

Anaplasmosis is a serious infection caused by ticks in cattle worldwide and causes high economic loss. Bovine anaplasmosis shows the symptoms of temperature, loss in weight, milk production decreased, whitish mucous membranes, jaundice, brownish urine, severe anaemia, hyper excitability abortion, and mortality without haemoglobinuria or haemoglobinuria. indirect antibody fluorescent test, a test of complement fixation or immunological medical test, Test of Agglutination, and indirect ELISA are examples of serological assays routinely used for *Anaplasma marginale* Sero-diagnosis [1].

In the nature, the wild animals were always considered for the existence and reappearance of zoonotic diseases. The most evolving zoonotic pathogens were originated by wild animals, The development and landscape modification and human activities are the main causes of the zoonotic

diseases, and the wild mammal host's ecosystem is also disturbed by these activities [2]. Anaplasmosis in cattle is a disease that occurs through infection of rickettsial hemoparasite, *Anaplasma marginale* in cattle and wild ruminants occurs through the bite of Ticks. In most of the areas of the world, this disease found including North and South America, African, The vector Caribbean, Russia, European countries bordering the Mediterranean, and the Middle and Far East. The vector of this protozoan parasite is *Ixodid* tick. And these are also spread involuntarily through flies fly or blood-sucking insects [3]. Diagnosis can be taking place through blood smear method in Giemsa stain, although this method is so convenient for the identification of a parasite in pre-symptomatic condition or carrier of the pathogen. At Present, (ELISA) the competitive enzyme-linked immunosorbent ass is one of the most used a diagnostic techniques for recognize the bovine *Anaplasma marginal*'s anti-major surface protein 5 (anti- MSP5) [4].

Bovine anaplasmosis is mainly caused by *Anaplasma marginale*, this pathogen is mostly prevalent in the Mediterranean basin. *Boophilus spp*., *Dermacentor spp*., *Hyalomma sp Ixodes ricinus and* , *Rhipicephalus* species of ticks are frequently convoluted in the spread of *Anaplasma marginale*, also insects and the intragenic pathway are involved in the transmission[5]. The prevalence of ticks has been increased world widely in humans and animals, perhaps due to changes in human activities, climatic conditions, demographics and land consumptions, resulting in the increase of infection rate and zoonotic diseases [6]. most prevalent in the world and is liable for cattle illness and death, Anaplasma *marginale* is the tick-borne pathogen in Brazil and other regions of Latin America including subtropical and tropical countries. *A. marginale* replicate within the parasitophorous vacuolar partition attached to erythrocyte membrane called obligate intracellular bacteria. Clinical disease outcomes from damage of host-infected erythrocytes and suffering from severe anemia.[7] Losses in the livestock industry attributed to ticks and TBDs are estimated to be between $ 14 billion and $ 19 billion globally, rickettsial TBDs are members of the Anaplasmataceae family and the leading causative agents of Anaplasmosis and ehrlichiosis. *Anaplasma marginal* is considered to be the most prevalent bovine TBD and bovine anaplasmosis occurs due to strong ticks of the genus Rhipicephalus (Boophilus) that mainly transmit A. marginal [8]. *Anaplasma marginal* (Rickettsiales: Anaplasmataceae) is intercellular pathogen termed by Sir Arnold Theiler in 1910, is globally endemic in tropical and subtropical areas. Cattle infection by A. marginal causes bovine anaplasmosis, a mild to severe hemolytic disease that causes significant economic losses to both the dairy industry and cattle meat. Spread of *A. marginal* to cattle occurs biologically by ticks and mechanically by stinging flies and blood-contaminated vapours. Male ticks and bovine hosts are consistently infected with A. marginal and act as reservoirs of infection.

[9]. Gram-negative obligatory in bacteria's group belongs to the *Anaplasma* genus having many members, e.g. *Anaplasma ovis, Anaplasma bovis*, and *Anaplasma marginale* [10]. Even though they are known as the pathogens who live inside the cells are called as intraerythrocytic, *Anaplasma phagocytophilum* and *Anaplasma bovis* attack the monocytes and neutrophil granulocytes respectively. *Anaplasma phagocytophilum* shows insignificant to severe febrile illness, as well as failure of organs and death occurs, *Anaplasma capra* is also *Anaplasma* sp. new zoonotic diseases, including other species of Anaplasma in the prokaryotic lists with permanent nomenclature (LPSN), but not legally published. [10].

Material and Methods

Collection of Samples (Ticks)

For the purpose of study parameters, Infested animals were selected for the collection of ticks, and these were stored in autoclaved glass vials and required information was label on these vials for recognition of the areas from where they were collected. Collection of Ticks were taken carefully so that the mouth parts were not cut off inside the animal body (a problem that may occur for secondary bacterial infection). Fine tweezers were used to collect the ticks. The

collected samples were identified at the Molecular Parasitology Laboratory of the Department of Veterinary Parasitology, Sindh Agricultural University. Tandojam, for identification of ticks genus (*Hyalomma, Amblyomma, Rhipicephalus, Boophilus*), the ticks were shifted in other autoclaved tubes containing preservative 70% ethyl alcohol.

Permanent Mounting of Ticks

This process was used for identification of ticks and formation of baseline data, the ticks were shifted from 70% ethanol and placed in the tubes having 10% potassium hydroxide (KOH) for the purpose of removing the exoskeleton (Scutum) during boiling for 10-20 minutes. These boiled ticks were further proceeded in the ethyl alcohol series of grades for 2 hours at 20%, 70%, 90%, and 100% for the purpose of dehydration. Finally, when these ticks were dehydrated they were placed at petri dish for washing purpose with water and then shifted into oil of clove for brightening purpose. These ticks (*Hyalomma, Amblyomma, Rhipicephalus, Boophilus*) were further proceeded for Incubation at room temperature for 24-48 hours for the purpose of that the muscles were be soft and shiny of the ticks.

Preparation of Potassium Hydro Oxide (KOH)

For the preparation of Potassium Hydro Oxide, 10 parts of KOH (Potassium hydro oxide) were dissolved in graded measuring cylinder (Borosil, Germany) having 90 cc of distilled water to acquire 10% Potassium hydro oxide (KOH) Solution.

Ethanol series making procedure

i. 20% ethanol

Ethyl alcohol absolute 100% was used with the quantity of 20 cc into 80 cc of distilled water.

ii. 70% ethanol

Absolute ethanol (100% Merck, Germany) was used with the quantity of 70 cc into 30 cc of distilled water.

iii. 90% ethanol

Absolute ethanol (100% Merck, Germany) was used with the quantity of 90 cc into 10 cc of distilled water.

iv. 100% ethanol

100% ethyl alcohol was used.

Permanent Mounting of Ticks on glass slide.

After the whole procedure the Ixodes ticks were set on slide and watched under dissecting microscope. their body parts were expanded with the help of pointer. Small amount of Mounting Media was set on the centre of the slide and tick was enclosed with cover slip in the middle for the permanent mounting of the ticks [11].

Identification of ticks

Ticks were recognized through the published keys. For that purpose, mouth parts location, capitulum location, genital aperture position, festoons and overall shapes of ticks were well-thought-out for the recognition as described by [12].

Blood Collection

Ticks carrying hosts were observed for blood sampling. 5 ml blood from each infested host was collected from jugular vein or ear vein from large and small animals respectively. The blood was collected using disposable syringe 23G*1&1/2" (Crespak Medical Industry Lahore). The blood transferred to EDTA containing tubes (BD Vacutainer K2 EDTA 3.6mg) and stored until further diagnosis of pathogen Viz. Blood Filming and DNA Extraction)

Blood Sampling Procedure

Hair removal was done from the collection site. To avoid any secondary infection in the sample the antiseptic (alcohol) is applied for disinfection with the help of cotton swab. A sterile needle was used to puncture blood vessel and blood is allowed to flow. Thin and thick blood smear was fixed to the stain in 70% alcohol to avoid erythrocyte rupture. While for collection of blood from the jugular vein, an injection was gently used, and 5 ml of blood was collected and preserved in EDTA containing tubes. These tubes were soaked by rolling around the palms because of the anti-coagulant mixture. The blood collection tubes were labelled with the owner's name, appearance. Location and other information about the host, sex, age and date of collection was obtained and recorded in a questionnaire specially designed for this purpose.

Nucleic Acid Extraction from Blood

Blood samples were taken for DNA extraction, commercial kit (Kit #K0722, GeneJET Genomic DNA purification, Thermo Scientific, USA) was used as per manufacturer's instructions.

Nucleic Acid Extraction from Ticks

Kit #K0722, GeneJET Genomic DNA purification, Thermo Scientific, USA was used as mentioned above. The procedure of nucleic acid extraction was same except that weight of ticks was measured 25-30 mg ticks were putted in TE buffer and then ticks were crushed with crushed machine. (XENOX Germany).

Method for Preparation of TE buffer

For preparation of TE buffer, 10 Mm of Tris were taken and 1 Mm of EDTA (Ethylene diamine tetra acetic acid) with the PH of 7.5. then it was dissolved in 100 ml of distilled water.

PCR Process

- 20 ul of TE Buffer was used for dilution of all primers.
- 25 µl of master mix was used to add in Primer (F) 8µl was added in a small tube (Neptune company).
- 25µl of master mix was used to add in Primer (R) 8µl was added in a small tube (Neptune company).
- 2ul of DNA extract was added in Primer (F) and Primer(R) individually.

  Table-1 displaying the constituents and measurements used in PCR process, In Thermal Cycler (Applied Bio- system, USA) the tubes filled with samples were inserted. The program was already set; Cap of machine was closed to start the program. The denaturation process of DNA was done at 94^{o}C for 5 min. 55^{0}C temperature was set for Annealing process for 1 min.72ºC temp: was set for preparing Two complementary copies of DNA from one DNA for I min, this program was started again from 94^{0}C. then we obtained the PCR product. After PCR the next procedure was electrophoresis.

Table 1: Constituents Used in PCR Process

Components	**Volume**
Master mix	25µl
Anaplasma marginale Primer(F)	8µl
Anaplasma marginale Primer(R)	8µl
Extracted DNA	2µl
Distilled water	7µl
Total	**50µl**

Table 2: Primers Used

Primers	Nucleotides	Species	References
***A.marginale*-F**	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	Anaplasma	[10]
***A.marginale* -R**	CCGGATCCTTAGCTGAACAGGAATCTTGC	Anaplasma	[10]

Making an Agarose Gel (1%)

1. Agarose powder 0.5g was measured and added into a 250mL conical flask 50mL of 0.5-0.6 X TAE buffer was used, and has been shaken to homogenized.
2. The material then subjected to place into oven for at least 1-2 minutes for dissolving purpose of the Agarose powder.
3. Boiled material then allowed to place on the table for cooling purpose for 5 minutes then the temperature was down from 60°C.
4. 2µL of ethidium bromide was used to add and then spins to mix.
5. Agarose gel is then allowing to cool for that reason to minimize production of vapours of ethidium bromide. The reason for allowing the Agarose to cool a little before this step was to minimize production of ethidium bromide vapor. ethidium bromide 10mg/mL solution was made up by using tablets and was placed at 4°C in the dark with the labelling of harmful or toxic.
6. Prepared Gel was put off calmly inside the electrophoresis tank. after few minutes the gel was checked by using a disposable tip and it was avoided from the formation of bubbles during poring off liquid form of agarose gel into the tank. Then the comb was inserted, and it was twice tested that the comb was positioned correctly.
7. 30 minutes were required to Gel became solidified from liquid form.
8. 0.5-0.6 X TAE was added inside the electrophoresis tank to sink the gel to 3-5mm deepness.

Sample loading

DNA ladder (Fermatas EU) was inserted before loading the samples in the gel in the very first well of agarose gel for the purpose of quantification of the size of the samples. Later, 4 µl of each sample was inserted in the remaining wells. when all samples were loaded then electrophoresis unit was allowed to start with 80 volts and 100 amperes for 30-45 minutes allowing samples to travel enough distance.

Gel Documentation

Later on electrophoresis was done, the Agarose gel was eliminated from the electrophoresis tank and gel was placed at the gel documentation system (Cleaver Scientific, Ltd, UK) for the purpose of visualization of the bands of DNA and to quantify their size by comparing their size with the ladder band size [13].

Results

This research was conducted for the purpose to investigate the molecular detection of *Anaplasma marginale* species in Cattle of different farm areas of Hyderabad and Mirpurkhas, Sindh, Pakistan. In the time of research, few animals were exposed infestation of ticks and indications of *Anaplasma*. Table -3 and figure-1 showing rate of Tick infestation in cattle was 540 (90%) at Mirpurkhas and 506 (84%) at Hyderabad, while rate of infection of *Anaplasma* was (88.33%) at Mirpurkhas and (65.38%) was at Hyderabad.

Beyond 1200 animals, 1046 (87.16%) animals were possessing ticks. Generally, Molecular detection of *Anaplasma marginale* was (77.67%) in different farm areas of Mirpurkhas and Hyderabad.

Table 3: The Rate of tick infestation and PCR detection of *Anaplasma marginale* in Cattle at Mirpurkhas and Hyderabad

Districts Hyderabad	CATTLE			
	Observed	Infested (%)	At Random blood samples	Infected samples (%)
Mirpurkhas	600	540 (90%)	300	265 (88.33%)
Hyderabad	600	506 (84%)	260	170 (65.38%)
Total	1200	1046 (87.16)	560	435 (77.67)

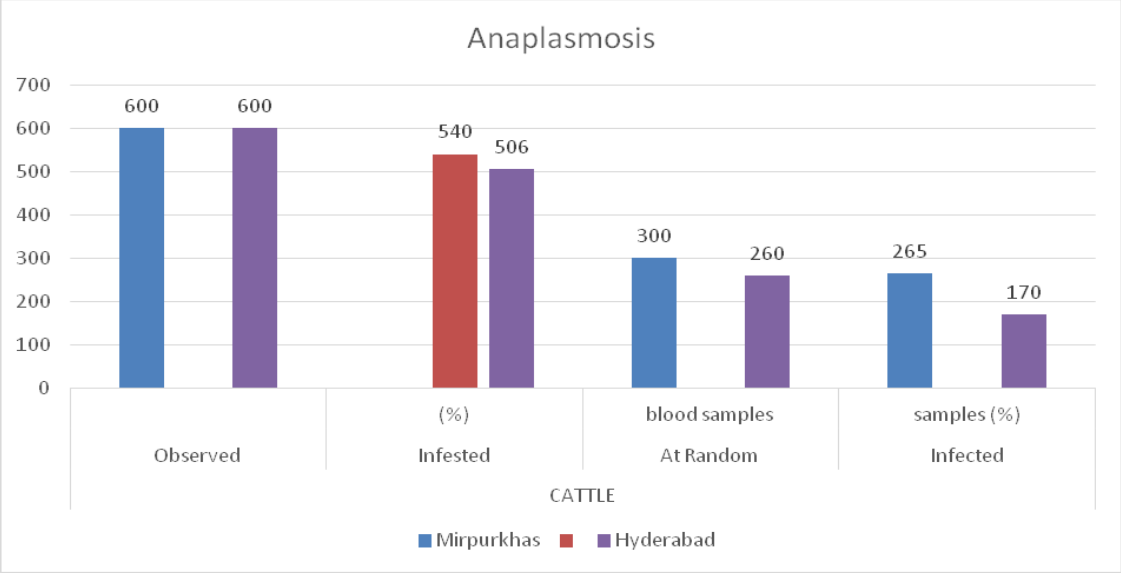

Figure 1: PCR detection of *Anaplasma marginale* in Cattle at Mirpurkhas and Hyderabad

Table- 4: Month-Wise (2020-2021) PCR detection from the samples of Mirpurkhas District

Month	Cattle		
	Total Animal Observed	Total Animal Infected	Percentage (%)
January	20	6	30
February	18	8	44.4
March	24	16	66.6
April	26	14	53.8
May	28	18	64.2
June	27	20	74.0
July	16	7	43.7
August	26	17	65.3
September	24	15	62.5
October	20	7	35
November	17	2	11.7
December	26	7	26.9
Total	272	137	50.36

Table-4 and figure-2 reveals the month-wise PCR detection from the blood samples of Cattle were taken from District Mirpurkhas which reveals the highest rate of infection in the month of June (74.0%) and the lowest rate of infection in the month of November.Table-5 and Figure-3 reveal the month wise rate of Anaplasmosis in Cattle at District Hyderabad.

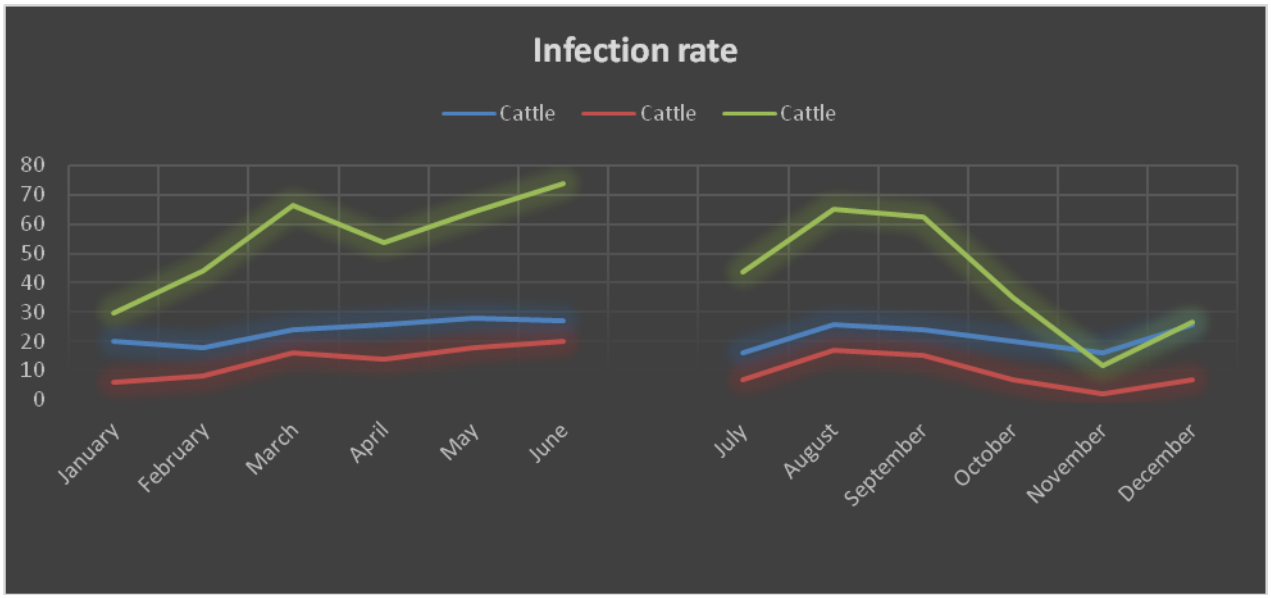

Figure-2: Month-Wise (2020-2021) PCR detection from the sample of Mirpurkhas District

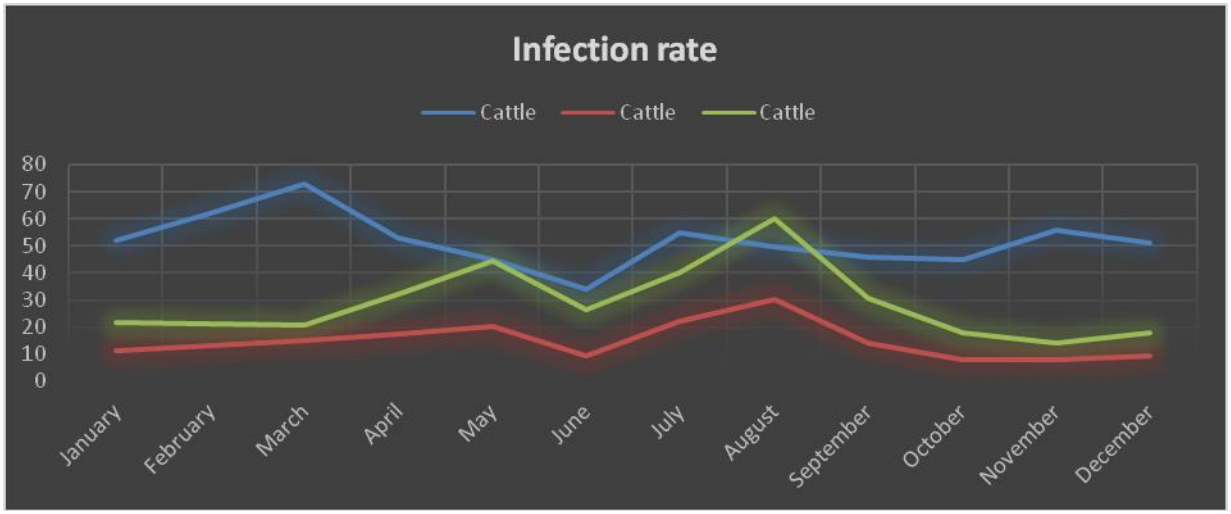

Figure 3: Month-Wise (2020-2021) PCR detection of *Anaplasma marginale* infection in Cattle at Hyderabad

The Table-6 showing the Tick Hyalomma species were found noticeable in the animals containing ticks and these ticks were dispersed on their whole body including their, ears, legs, externally on their reproductive organs and udder. Position of the sites where the ticks found mostly in cattle specified with tick infestation were external reproductive organs, perineum and udder, these ticks genus were *Hyalomma* (26.11%), followed by *Amblyomma* (25.37%), *Boophilus* (24.62%), *Rhipicephalus* (23.88%). Figure-4 Showing the DNA bands at 405 and 290 base pairs in Gel Electrophoresis of Amplified PCR Product of *Anaplasma marginal*

Discussion

Our findings of present study are very much correlated with those of [14] who reported that analysis was executed by Kaliobia governorate Egypt on cattle with the age from 1- 7 years with the total no 100, consequence of the disease increase with their age, they were also done the Molecular detection of parasite *Anaplasma marginale* through Polymerase Chain Reaction from the blood samples got from cattle possesses ticks. For the detection of *Anaplasma marginale* particularly PCR using primers derived from msp5 gene, assay worked primers was particularly for the gene encoding. Different authors [15] have reported livestock of Boeen Zahra and respectively. In different topographic areas Hard ticks compositions were different. Commonly *Hyalomma* species had originate in that areas. The study was convoyed at India of Mathura region by [16] was main focused on the financial influence of various ticks species on cattle, The survey was carried out for the epidemiologically demonstration of most commonly found ticks species and infestation rate during the period of July 2010 to July 2011 in Indian zebu cattle. Total 2,515 zebu cattle were inspected randomly all over the year at different areas. The overall rate of ticks infestation in cattle was 60.07 %. The minimum occurrence was in January 46.07 %, The maximum incidence was investigated in September 75 %. Different authors [17] have reported about the tick infestation in sheep having highest rate of infestation 17.8% , Although in camels no tick infestation found. The mostly found tick's species that causes disease in ruminants was *Boophilus annulatus* 26.5% followed by *Hyalomma anatolicum* 6.1% then *Rhipicephalus turanicus* 3.4%. Regarding the related threat, tick species found statistically significant ($P < 0.05$), as the highly infestation were found in Friesian cow 77.5%, Above 3 years 78.8% surveyed by at age, 2 months (57.8%) and during hot season infestation found highly significant ($P < 0.01$) in cattle 76.5% surveyed by goats and sheep 33.3% & 22.9% respectively in correlation with the results of tick infestation in winter.

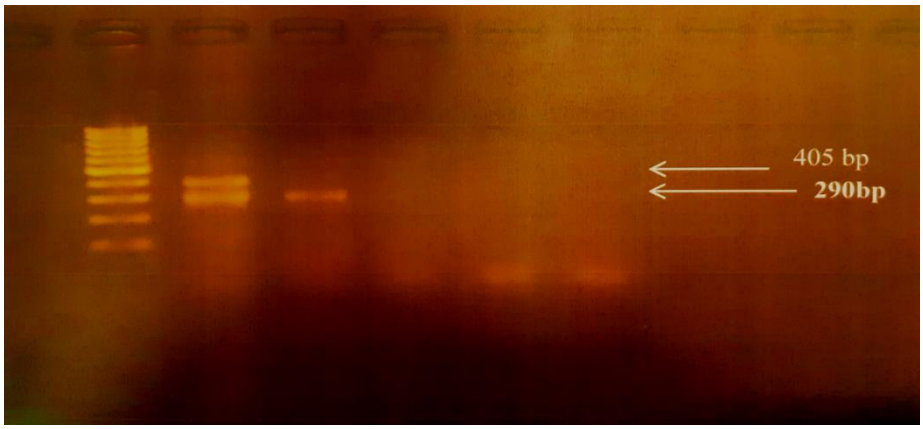

Figure-4. Showing the DNA bands at 405 and 290 base pairs in Gel Electrophoresis of Amplified PCR Product of *Anaplasma marginal*

Table- 5: Month-Wise (2020-2021) PCR detection from the samples of Hyderabad District

Month	Cattle		
	Total Animal Observed	**Total Animal Infected**	**Percentage(%)**
January	52	11	21.5
February	62	13	20.96
March	73	15	20.54
April	53	17	32.0
May	45	20	44.4
June	34	9	26.4

July	55	22	40.00
August	50	30	60
September	46	14	30.43
October	45	8	17.77
November	56	8	14.28
December	51	9	17.64
Total	622	176	28.29

Table 6: Different Identified Genera of ticks in Cattle and their infestation rate.

Animal species	Amblyomma	Boophilus	Hyalomma	Rhipicephalus
Cattle	34(25.37 %)	33(24.62%)	35(26.11%)	32(23.88%)

Conclusions

Based on findings of this study of Molecular detection *Anaplasma marginale* the infection rate was higher in District Mirpurkhas and lower in District Hyderabad. Maximum infested ticks were concerned to genus *Hyalomma*, Month wise data exposes the maximum rate of infection in summer season (June) and lowermost rate in the winter season (November) at District Mirpurkhas, while at Hyderabad, the month wise data indicates the uppermost rate in the hot season (May) and lowermost rate in cool season (November).

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

This research did not receive any specific funding from agencies in the public, commercial or not-for-profit sectors. This is the part of the study arose from my PhD data which I had collected.

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENTS

None

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