Research article

RICKETTSIAL AGENTS DETECTION FROM BLOOD OF TICK-INFESTED ANIMALS IN LOWER SINDH- COMPARISON OF CONVENTIONAL AND MOLECULAR APPROACH

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ABSTRACT

Ticks are important vectors of human and animal pathogens. They are considered as main vectors for transmission of rickettsial agents affecting animal and human health. The study was designed to investigate district wise pattern and detection of rickettsial agents by using molecular and conventional techniques in blood samples of infected cattles, buffalos, sheep and goats. A survey study was carried out in lower Sindh (Tharparkar, Badin, Hyderabad, Karachi, Tando Muhammad khan, Thatta and Mirpurkhas). Blood samples were collected randomly from infected Cattles, buffalos, sheep and goats and transported to the Molecular Parasitology laboratory, Sindh Agriculture University, Tandojam, followed by examinations under stereomicroscope and Polymerase Chain Reaction (PCR). The study showed that overall infection of Rickettsial agents among infected animals was recorded following Microscopy/ Blood smear test in cattles, buffalos, sheep and goats was 41.79, 49.09, 46 and 41.66% respectively, whereas overall infection through PCR in cattle, buffalo, sheep and goat was 39.55, 43.55, 46 and 55.55% respectively. Whereas animal-wise data through PCR indicates that in case of Goats (55.55%) were more susceptible to rickettsial infection as compared to sheep (46%), buffaloes (43.55%) and cattle (39.55%). The highest rate of rickettsial agents was found in district Tharparkar and lowest rate was found in district Karachi. Microscopy/Blood smear method indicates that Buffaloes were more susceptible for infection. Whereas PCR indicates Goats were more susceptible for infection.

Key Words: Ticks (Vectors), Rickettsial agents (Pathogens), Molecular and Conventional techniques, Lower Sindh

INTRODUCTION

Ticks are potential vectors and reservoirs of many infectious agents such as. Pasteurella multocida, brucella abortus and salmonella typhimurium in both humans as well as animals (1). Ticks have enormous capability to adapt to changing geoclimatic conditions and can therefore expand their distribution range (2). They are known as main vectors for transmission of many pathogens such as viral, bacterial, rickettsial and parasitic infestations (3). Till date 899 species of ticks are known which belong to three families, namely Ixodidae, Argasidae and Nuttalliellidae (represented by a mono- typic species restricted to South Africa (4). After sucking blood, the outer surface of a tick grows to 200-600 times as compared to its unfed body weight (5). Prevalence of tick-borne pathogens (TBPs) and their occurrence in bovines have been found all over the Pakistan (6). Hyalomma anatolicum transmitted some of tick-borne pathogens (TBP) which have zoonotic importance (e.g. Crimean Congo haemorrhagic fever) (6). Generally, rickettsioses is the term used for those diseases which have continuous spectrum of severity of illness and overlapping clinical manifestations. *R. rickettsii*, *R. prowazekii*, *R. conorii*, and *R. typhi* are rickettsial agents with a potential to cause life-threatening diseases (7). The main cause of granulocytic anaplasmosis is Anaplasma phagocytophilum which is considered as one of the most important species from humans' point of view because of its zoonotic potential.

Ticks are known as etiological agents of tick-borne fever in ruminants and equine, canine and human granulocytic anaplasmosis (EGA, CGA and HGA, respectively) (8). Genetic diversity has been recognized among various European strains of *A. phagocytophilum* shown through phylogenetically analysis of genes such as groEL (chaperone protein encoding gene) (8) Rickettsiae are commonly defined as genetically related, obligatory intracellular bacteria that reside in an arthropod host during a part of their zoonotic cycle. (9) Ticks as parasites are vectors of many important human and animal pathogens such as Q fever Babesiosis, tick paralysis, haemorrhagic fever, Lyme disease (LD), tick-borne encephalitis and tick-borne muscular fever. Rocky mountain spotted fever, which is caused by Rickettsia rickettsii, is a life-threatening, tick-borne disease that occurs throughout much of the United States (11). It has been estimated that 10% of the known tick species act as vectors of the pathogens of above mentioned diseases (12). They also pose a great threat to global animal production in terms of economic expenditure incurred through treatment of various inflammatory and hematologic conditions that occurred in humans and animals through these tick-borne diseases. It has further been suggested that around 80% of cattle production worldwide is at increased risk of tick-borne infections. (6) Rickettsia have a comparatively small genome developed though reductive evolution because of their dependence on the host for survival and to carry out essential functions. (18) The genomes of various species of rickettsia have been sequenced such as *Rickettsia prowazekii* and *Rickettsia conorii* (19). There was limited literature available looking at the presence of rickettsial organisms in ticks in lower Sindh. Therefore, this study was aimed to investigate district wise pattern and compare detection of rickettsial agents by using molecular and conventional techniques in blood samples of infected cattles, buffalos, sheep and goats.

METHODOLOGY

A survey was conducted in lower Sindh including Tharparkar, Badin, Hyderabad, Karachi, Tando Muhammad khan, Thatta and Mirpurkhas districts. Blood samples were collected randomly from infected Cattle, buffalos, sheep and goats and transported to the Molecular Parasitology laboratory, Sindh Agriculture University, Tandojam, followed by examinations under stereomicroscope and Polymerase Chain Reaction (PCR)(24).

Blood Collection

Host that carried ticks were selected 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 transferred to Ethylene diamine tetra acetic acid (EDTA) containing tubes and stored until further diagnosis of pathogen (Viz. Blood Filming and DNA Extraction) was carried out.

Blood Sampling Procedure

Hairs from collection site were removed using automatic hair shaver. Cotton swab soaked in the antiseptic (alcohol) was applied for disinfection to avoid any secondary contamination in the sample. Ear vein was gently punctured with sterilized needle and blood was allowed to ooze out. A thin and thick blood smear was prepared fixed on spot in 70% alcohol to avoid rupturing of erythrocytes. In case of collection from jugular vein, syringe was gently used and 5ml of blood was drawn and preserved in EDTA tubes. The blood vials were soaked by rotating between palms of two hands for proper mixing of the anti-coagulant. The collection tubes were labelled with the name of owner, type of host and refrigerated at -20° C. Relevant information on host, sex, age and date of collection was obtained and recorded on a Proformma specifically designed for this project.

Blood Smear Method

Two methods were applied for blood examination viz. thin and thick blood smear(s).

Thin Smear method

For making thin blood smear, a glass slide was dipped in 95% alcohol. About 2ul of blood was placed on one end of the slide (called microscopic slide). Another slide (called spreader slide) was placed on microscopic

slide containing the droplet of blood, positioning it about an inch in front of the droplet. The spreader slide was quickly run on the surface of microscopic slide at angle of 45 degrees. In a smooth motion, the spreader slide was pushed forward to spread the blood in a layer. Prepared blood slide was allowed to air dry for one minute and fixed in absolute alcohol for 5 minutes. Slides were removed from alcohol jars and air dried. Dried slides were stained in freshly prepared Romanowisky stain (commonly called Giemsa's stain) for 5 minutes.

Thick Smear method

Procedure for making thick blood smear was same except that the spreader slide was moved slowly to make a thick film on microscopic slide.

Nucleic Acid Extraction from Blood

DNA was extracted and obtained from collected blood by commercial kit (GeneJET Genomic DNA purification Kit #K0722, Thermo Scientific, USA) as per manufacturer's instructions. 20 µl of Proteinase-K solution and 400 µl of lysis solution were added to 200 µl of whole blood. The mixture was mixed by vortexing in order to obtain a uniform suspension. It was then kept in incubation at 56° C for around 10 minutes or till the cells were completely hemolysed. Afterwards, ethanol in a quantity of 200 µl was added and vortexed. The solution obtained was then transferred to GeneJET genomic purification column and was centrifuged at 6000xg for up to one minute. The flow through solution in the collection tube was discarded whereas purification column was transferred in a new collection tube. Wash buffer (500 µl) was added to this collection tube which was then centrifuged at 8000xg for one minute. The flow through solution was again discarded while transferring the purification column to a new collection tube to which 500 µl of wash buffer 2 (with ethanol already added) was added and further centrifuged at 12000xg for three minutes. The purification column was transferred into 1.5ml micro tube whereas collection tube containing flow through solution was again discarded. 200 µl of elution buffer was added to 1.5 ml micro tube containing purification column and it was then incubated at room temperature for two minutes and then centrifuged at 8000xg for one minute. The DNA thus extracted is obtained by discarding the supernatant and its concentration was evaluated by spectrophotometer (Thermo scientific Nano drop 1000).

PCR Process

Table-1 shows components and volume used in PCR process, the sample tubes were loaded in Thermal cycles (Applied Bio- system, USA). The cycles were already set. The lid of machine was closed to start the operation. DNA was denatured at 94oC for 5 min. Annealing process took place at 550C for 1 min. Two complementary copies of DNA were obtained from one DNA at 72^{0}C for I min, the cycle again started from 940C. The PCR product was subjected to electrophoresis.

Table -1 Components used in PCR process.

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

Method

All primers were diluted with 20ul of TE Buffer. Piro primer (F) =8µl was added in 25 µl of master mix in a small tube (Neptune company). Piro primer (R) =8µl was added in 25 µl of master mix in a small tube (Neptune company). 2µl of DNA extract were added in Piro (F) and (R) primers respectively.

Agarose Gel (1%)

Agarose gel powder was taken in a quantity of 0.5 grams in a conical flask. 50 ml of 0.5 TAE buffer was then added to the agarose powder and microwaved for about one minute in order to dissolve the powder. It was then allowed to cool down to 60° C. afterwards, 2 µl of ethidium bromide was added to gel solution and it was then poured down slowly into the tank. Comb was correctly positioned in the tank and it was then left for at least 30 minutes to solidify. Before using the gel, it was submerged in 0.5 TAE buffer in the tank.

Table-2 Primers used

Primers	Nucleotides	Species	References
PIRO-F	AATACCCAATCCTGACACAGGG	All Piroplasms	Karimi *et al*.,2012
PIRO-R	TTAAATACGAATGCCCCCAAC	All Piroplasms	Karimi *et al*.,2012
Bi-F	AATAACAATACAGGGCTTTCGTCT	Babesia bigemina	Kim *et al*., 2007
Bi-R	ACGCGAGGCTGAAATACAAC	Babesia bigemina	Kim *et al*., 2007
***T. annulata*-F**	CACCTTCGACAAGAAAGAAGTCGG	Theileria	Designed in Mather lab, USA
***T.annulata*-R**	TGAGAAGACGATGAGTACTGAGGC	Theileria	Designed in Mather lab, USA

Sample loading

Before loading the samples in the gel, DNA ladder (Fermantas EU) was loaded down in the very first well of agarose gel in order to quantify the size of the samples. Afterwards, 4 µl of each sample was loaded in the subsequent wells. After all samples have been added, electrophoresis unit was allowed to run with 80 volts and 100 amperes for 30-45 minutes allowing samples to travel a sufficient distance.

Gel Documentation

After electrophoresing the samples, the gel was removed and put in gel documentation system (Cleaver Scientific, Ltd, UK) in order to visualized the bands of samples and to determine their size by comparing them with the ladder.

Statistical methods

Statistical package for Social Sciences (SPSS Version 21) was used for results analysis. Frequencies and percentages were analysed and presented.

RESULTS

Blood samples were collected from different districts of lower Sindh, in order to compare sensitivity of polymerase chain reaction with that of blood smear method, Table-3 and 4 reveals the detection of rickettsial agents through blood smear method then blood samples were subjected to PCR for detection of rickettsial infection. Table-5,6 and 7 reveals that PCR is more sensitive diagnostic method as samples that were negative via blood smear method were found positive when diagnosed through PCR. Presence of rickettsial agents through blood smear test was confirmed under high power magnification, whereas through PCR the detec-

tion was confirmed by looking at the bands that appeared at 405, 150, 170 and 290 base pairs on gel documentation. (Figure-1-3), Table- 3 to 6 shows data on Cattle, Buffalo, Sheep and Goat that were diagnosed positive for rickettsial agents via blood smear test and PCR, the highest ratio of differences in districts was found in District Tharparkar 83:83, 81:87, 66:66, 57:71 in cattle, buffalo, sheep and goat, whereas lowest ratio of differences is found in district Karachi was 36:30, 45:41, 25:25, 28:42 in cattle, buffalo, sheep and goat respectively.

Table 3. Detection of rickettsial agents in Cattle and Buffalo through blood smear method in lower Sindh

Districts	CATTLE				BUFFALO			
	Observed	Infested	Random blood samples	Infected samples (%)	Observed	Infested	Random blood samples	Infected samples%
Karachi	96	33	12	36.36	117	24	11	45.83
Hyderabad	27	20	8	40	67	33	14	42.42
Badin	27	17	6	35.29	26	15	7	46.66
Tharparkar	10	6	5	83.33	20	16	13	81.25
T.M Khan	59	24	10	41.66	119	35	16	45.71
Mirpurkhas	37	24	11	45.83	73	29	14	48.27
Thatta	16	10	4	40.00	44	11	5	45.45
Total	272	134	56	41.79	466	163	80	49.07

Table 4. Detection of rickettsial agents in Sheep and Goat through blood smear method in lower Sindh

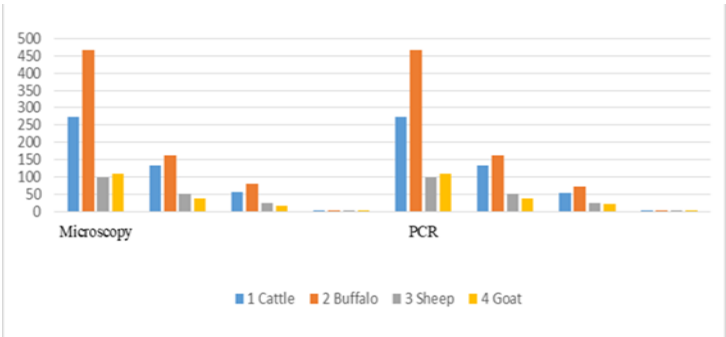
Districts	SHEEP				GOAT			
	Observed	Infested	Random blood samples	Infected samples %	Observed	Infested	Random blood samples	Infected samples %
Karachi	11	4	1	25	32	7	2	28.57
Hyderabad	15	6	2	33.33	10	3	1	33.33
Badin	18	14	5	35.71	16	5	2	40
Tharparkar	12	3	2	66.66	13	7	4	57.14
T.M Khan	15	5	2	40.00	16	5	2	40
Mirpurkhas	9	3	1	33.33	12	4	2	50.00
Thatta	14	4	2	50.00	10	5	2	40.00
Total	94	39	15	38.46	109	36	15	41.66

Table 5. Detection of rickettsial agents in Cattle and Buffalo through PCR in lower Sindh

Districts	CATTLE				BUFFALO			
	Observed	Infested	At Random blood samples	PCR detection(%)	Observed	Infested	At Random blood samples	PCR detection%
Karachi	27	20	6	30	67	33	9	27.27
Hyderabad	96	33	10	30.30	117	24	10	41.66
Badin	27	17	7	41.17	26	15	6	40
Tharparkar	10	6	5	83.33	20	16	14	87.5
T.M Khan	59	24	9	37.5	119	35	13	37.14
Mirpurkhas	37	24	10	41.66	73	29	12	41.37
Thatta	16	10	6	60.00	44	11	7	63.63
Total	272	134	53	39.55	466	163	71	43.55

Table 6. Detection of rickettsial agents in Sheep and Goat through PCR in lower Sindh

Districts	SHEEP				GOAT			
	Observed	Infested	At Random blood samples	PCR detection%	Observed	Infested	At Random blood samples	PCR detection%
Karachi	11	4	1	25	10	3	1	33.33
Hyderabad	15	6	2	33.33	32	7	3	42.85
Badin	18	14	5	35.71	16	5	3	60
Tharparkar	12	3	2	66.66	13	7	5	71.42
T.M Khan	15	5	2	40.00	16	5	3	60
Mirpurkhas	9	3	1	33.33	12	4	2	50.00
Thatta	14	4	2	50.00	10	5	3	60.00
Total	94	39	15	38.46	109	36	20	55.55



Graph-1 showing the comparison between Microscopy and PCR

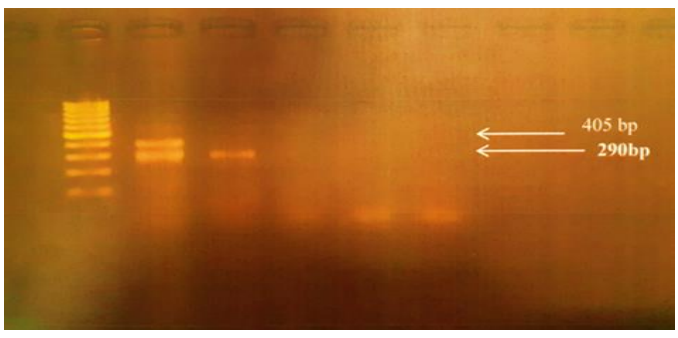


Figure-1 Gel electrophoresis of amplified PCR product of rickettsial agents of buffalo blood DNA

Table-7 shows the pooled data of all districts indicates that through blood smear test Buffaloes were (49.09%) more susceptible to rickettsial infection as compared to sheep (46%), cattle (41.79%) and goats (41.66%), Whereas through PCR, the data indicates that in case of goats 55.55% were more susceptible to rickettsial infection as compared to sheep (46%), Buffaloes (43.55) and cattle (39.55%).

Table 7. Pure and mixed infection of rickettsial agents in cattle, buffalo, sheep and goat

S. No.	Name Of Animal	Microscopy				PCR			
		Total no. of Animals observed	Total no. of animals infested	At Random blood samples	% of infected samples through Smear method	Total no. of Animals observed	Total no. of animals infested	At Random blood samples	% of infected samples through PCR
01	Cattle	272	134	56	41.79%	272	134	53	39.55%
02	Buffalo	466	163	80	49.09%	466	163	71	43.55%
03	Sheep	99	50	23	46%	99	50	23	46%
04	Goat	109	36	15	41.66%	109	36	20	55.55%
Total		946	383	174	45.43%	946	383	167	43.60%

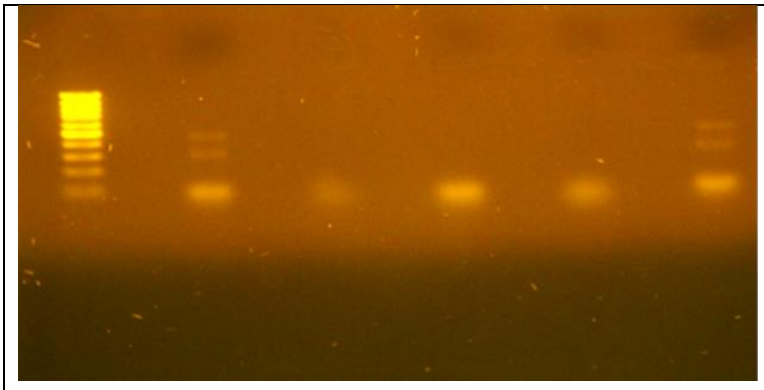


Figure-2 Gel electrophoresis of amplified PCR product of rickettsial agents of Cattle blood DNA

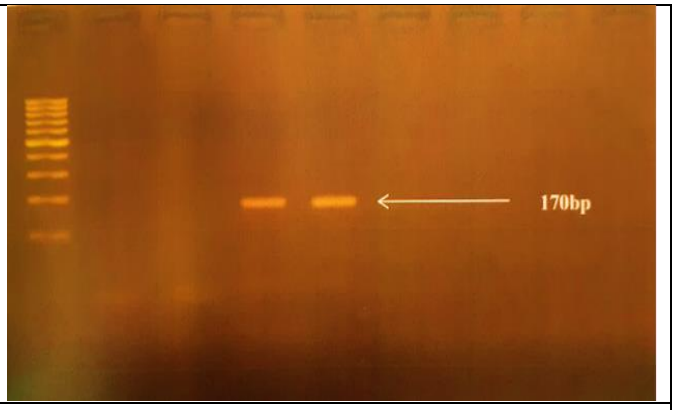


Figure-3 Gel electrophoresis of amplified PCR product of rickettsia agents of Buffalo blood DNA

DISCUSSION

The blood samples of tick-carrying cattle, buffalo, sheep and goat conventionally confirmed blood samples were subjected for PCR detection of Piroplasms, for this purpose, DNA was extracted from positive blood samples and quantified on Nano-drop spectrophotometer.

Primers used for PCR reaction are described in Table 2. Different concentrations of $MgCl_2$ were used for PCR reaction i.e. 5ul & 6 ul for *T. annulata* whereas for *B. bovis* and *B. bigemina* concentration of $MgCl_2$ was 3ul. PCR was done for 30 cycles with following conditions: Denaturation at 94° C for 5 min, 94° C for 30 sec. Temperature was lowered for several minutes to allow both forward and backward (right or left) primers to anneal with the complementary sequences. At this stage three conditions 50° C, 55° C and 60° C for 30 secs

were checked for each primer set. Finally, extension was carried out 72°C for 45 secs. Analysis of amplified product by electrophoresis was done with a 1% agarose gel.

The results were photographed with Gel Documentation System (Gel Doc USA). In order to compare sensitivity of polymerase chain reaction, with that of blood smear method, blood samples were subjected to PCR detection of rickettsial infection. (2) gave findings take out at Maharashtra (India) by (3) *Boophilus*, *Haemaphysalis*, *Hyalomma, Amblyomma, Nosoma* and *Rhipicephalus* were found tick infesting in subfamily Bovinae animals, which includes cattle, buffalo, and kudus at 40, 16.96, 20.14, 10.22, 4.56, and 1.96 percent attentiveness, correspondingly. He discovered 8 different tick genera of ticks to be precise as *Boophilus, Rhipicephalus, Hyalomma, Amblyomma, Dermacentor, Haemaphysalis, Ixodes,* and *Aponoma* from many segments of Pakistan. The lessen quantity of the genera perceived possibly would be the reason of looked-for the partial region stipulated for the present investigation, also in a partial investigation takeout by (4) and (5) stated 4 genera, even if dissimilar from every one, for the tick troublesome invasion resident of a tract of land on which crops and often livestock are raised for livelihood in their particular investigation.

CONCLUSION

Information regarding to cattle, buffalo, sheep and goat farms (946 observed animals out of which 383 were the infested animals and 45.43% were infected animals through Microscopy and 43.60% were infected through PCR) According to Microscopy Buffaloes were more susceptible to rickettsial infection as compared to cattle, sheep and goat. According to PCR Goats were more susceptible to rickettsial infection as compared to cattle, buffalo and sheep, Highest rate of rickettsial infection is found in district Tharparkar. Lowest rate of rickettsial infection is found in district Karachi.

Ethical Consideration: The study was approved by the Ethical committee of xxxx

Conflict of Interest: There is no conflict of interest.

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