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Molecular, Hematological and Biochemical Investigation of *Trypanosoma* spp. in Sheep

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ABSTRACT

Background: *Trypanosoma* spp. is a flagellated unicellular protozoan parasite which caused a disease known as trypanosomiasis in various animals among many areas worldwide resulting in severe economic losses due to morbidities and mortalities. This study was conducted to estimate the prevalence of *Trypanosoma* spp. in sheep using the molecular assay with detection the impact of infection on various risk factors and blood markers.

Methods: Totally, 341 sheep were selected from different areas in Wasit province (Iraq) during June to September (2023) and subjected to draining the venous blood that tested molecularly by the conventional polymerase chain reaction (PCR). Status of different hematological, biochemical and mineral markers were further investigated.

Result: Targeting *16S sRNA* gene, 7.92% animals were positively infected with trypanosomiasis. Relation to risk factors, positive infection and risk were significantly higher in females than males and in sheep aged >1-3 years than others. Hematological findings reported a significant reduction in RBCs and Hb; while biochemically, significant elevation was reported in concentration of urea. Regarding various minerals, insignificant alteration was seen in infected sheep when compared to healthy ones.

Key words: *16S sRNA* gene, Mineral, Polymerase chain reaction, Trypanosomiasis.

INTRODUCTION

Trypanosoma belongs to *Trypanosomatida* Family of *Kinetoplastea* Order, which first diagnosed in Indian camels by Griffith Evans in 1880 (Eyob and Matios, 2013; Borges *et al*., 2021). Then, many domestic animals were found to be incidentally infected with trypanosomiasis including *T. congolense* in goats, cattle, sheep, horses and dogs; *T. vivax* for cattle, horse, sheep and goats; and *T. brucei* and *T. evansi* for all mentioned animals as well as camels. Different studies were carried out attempting to clear the higher risk to reinfection with trypanosomiasis in an endemic farms (Auty *et al.,* 2015; Vourchakbe *et al*., 2023). One of the most important key factors is the survival mechanism that developed by this parasite to escape the host immune response is through antigenic variation and exhibiting on various membrane surface glycoproteins (Cardoso *et al*., 2016; Pech-Canul *et al*., 2017).

Consequently, this mechanism makes some of the animals in the infected herds act as a reservoir and reemerging of trypanosomiasis (Jilo *et al*., 2017). Whenever, the great risk of infection is primarily related to the existence of nearby wildlife reservoir such as bushbuck (*Tragelaphus scriptus*), warthog (*Phacohoerus aethiopicus*), bush pig (*Potamochoerus porcus*), lion (*Panthera leo*), white rhinoceros (*Ceratotherium simum*), leopard (*Panthera pardus*) and wild Equidae as well as hematophagous flies (stable flies, *Tabanids* and *Stomoxys*) that transmit the disease (Maharana *et al*., 2019; Eid *et al*., 2023; Agrawal *et al*., 2024).

The transmission of infection to various animals can occur *via* several routes; however, the most commonly is the cyclical transmission when the parasite is inoculated with saliva of infected tsetse flies (Abdi *et al*., 2017). Other routes are the mechanical transmission by biting insects especially when interrupting during feeding and intrauterine during partum by vertical transmission (Desquesnes *et al*., 2013). Oral transmission of trypanosomiasis may also occur particularly after birth with *T. brucei* when calved animal ingested contaminated blood and fluid during parturient; however, this route also reported in carnivores when fresh infected prey is eaten (Fagiolo *et al*., 2005; Salman and Steneroden, 2014). The high risk of re-infection and outbreak occurrence is usually differently related to the prevalence of vectors in the endemic area which depends mostly on the climate factor-like temperature and humidity in tropical regions that are favorable for vector breeding all year seasons (Liu and Zhou, 2015; Maharana *et al*., 2016). Also, the seasonal influence is related to activity of vectors climate changes outcome (Booth, 2018).

Since many factors may impact on the prevalence of trypanosomiasis including ecological zones, type of

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husbandry practices, the affected breeds and applied to control regulates (Meyer *et al*., 2016; Agrawal *et al*., 2020). In Iraq, sheep trypanosomiasis was largely neglected (Mahmood and Alobaidii, 2022); therefore, this study was conducted to estimate the prevalence of *Trypanosoma* spp. in sheep using the molecular assay with detection the impact of infection on various risk factors and blood markers.

MATERIALS AND METHODS

Samples

An overall 341 sheep of both sexes and different ages were selected to the current study at different areas of Wasit province (Iraq) during June to September (2023). Each study animal was subjected to collection 5 ml of jugular venous blood into an EDTA-K_2 vacutainer plastic tube (AFCO, Jordan). At laboratory Unite (College of Veterinary Medicine, University of Wasit), blood samples were examined hematologically and then centrifuged (3000 rpm/5 minutes) for biochemical and mineral markers.

Hematology

Automated hematological analysis was performed by Mythic 18-Vet Analyser (Orphee SA, Switzerland) to obtain the main hematological parameters (Gharban and Yousif, 2020). Total red blood corpuscles (RBCs) count, hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cells (WBCs) count, lymphocytes (L), monocytes (M), neutrophils (N), eosinophils (E) and basophils (B) were measured. The steps of analysis were applied in based on manufacturer instruction. The values of lymphocytes/ neutrophils (L/N) ratios were measured manually.

Biochemical markers

Serum samples of all study camels were analyzed using of specified kits (Biomaghreb/Tunisia) to measurement the total protein, albumin, globulin, blood urea, creatinine, cholesterol, glucose and total bilirubin; while albumin/ globulin (A/G) ratio was calculated based on obtained results.

Minerals

Iron (Fe) was determined by the atomic absorption spectrophotometery using a commercial available kit (SunLong Biotech/China); while other minerals including serum calcium (Ca), chloride (Cl), magnesium (Mg), phosphorus (P), sodium (Na) and potassium (K) were measured according to manufacturer instructions of each specific colorimetric assay kits (Linear / Spain).

Molecular study

Following the manufacturer instructions of the G-spinTM Total DNA Extraction Kit (Intron Biotechnology, South Korea), the frozen blood samples were thawed, processed and the extracted DNAs were tested for quantification of purity and concentration by the NanoDrop Spectrophotometer System (Thermo Scientific, USA). Targeting *18S rRNA* gene, one set of primers was designed [F: (5′- GGG AAT ATC CTC AGC ACG TT-3′) and R: (5′- ACT GGG CAG CTT GGA TCT C-3′)] based on the GenBank Iranian isolate (Ref. No. KC427089.1), the MasterMix tubes of the Accupower® PCR Premix Kit (Bioneer, South Korea) were prepared at 20 µl and subjected to Thermal Cycler conditions as following: 1 cycle initial denaturation (95°C, 5 minutes), 35 cycles of denaturation (95°C, 30 seconds), annealing (58°C, 30 seconds) and extension (72°C, 1 minute) and 1 cycle final extension (72°C, 5 minute). PCR products were analyzed by electrophoresis of agarose-gel stained with Ethidium Bromide at 80 Am and 100 Volt for 90 minutes. Using the UV Transilluminator System (Clinx, China), the positive samples were detected at a product size of 300 bp.

Statistical analysis

All study results of hematology, biochemical markers, minerals and coagulation factors were analyzed by the *t*-test (GraphPad Prism Software), to identify significant variation at $P<0.05$ (*) and $P<0.01$ (**) between the infected and healthy groups. Study values recorded as either percentage (%) or Mean ± Standard Error (M±SE), (Gharban, 2022).

RESULTS AND DISCUSSION

Molecular testing

Of 341 sheep selected, 37 (10.85%) animals were showed a positive result to *Trypanosoma* spp (Fig 1, 2). In endemic areas, *Trypanosoma* spp. considered as one the major veterinary problems, worldwide.

In this study, 10.85% of sheep were infected with *Trypanosoma* spp. using the conventional PCR assay; which comparatively less than reported recently in Iraq (21.04%) by Mahmood and Alobaidii (2022). Globally, the prevalence of sheep trypanosomiasis was 1.3-1.7% in Algeria (Benfodil *et al*., 2020), 2% in Ecuador (Coello-Peralta *et al*., 2020), 8.3% in Somalia (Hassan-Kadle *et al*., 2020), 24.3% in Chad (Vourchakbé *et al*., 2020) and 28.3% in Brazil (de Araújo-Neto *et al*., 2023). Random selection of study sheep grazing at different areas, ages and sexes might explain variation between the results of these studies. Also, the availability of suitable medication at some areas with the availability of awareness towards chemoprophylaxis and providing of good feeding might be considered as additional reasons for this difference. However, the susceptibility of sheep to *Trypanosoma* is conflicted between studies according to the species. Some authors mentioned that sheep are susceptible to *T. vivax*, *T. evansi* and *T. equiperdum* (Parra-Gimenez and Reyna-Bello, 2019); while others reported that sheep are susceptible to *T. brucei* (Vourchakbé *et al*., 2020) and *T. cruzi* (de Araújo-Neto *et al*., 2023).

Risk factors

Relation to sex, values of positivity and risk were significantly higher in females than males; while for age, values of positivity and risk were increased significantly in sheep of

>1-3 years old when compared to others; 6 months, 6-12 months, >3-4 years and >4 years old (Table 1). The factors of age and sex remain the main factors known to be affected the prevalence of any infection among different animal species. Reducing of maternal immunity after 6-12 months, grazing of animals in pasture and exposure to vector/ contaminants, as well increasing of stress due to nutritional deficiency and maturity could explain the high incidence of infection after 1 year of age. The absence of positivity in study lambs of <6 months could result by either maternal immunity or high attention by owners due to the high economic benefits. Decreased susceptibility of adult population compared to younger could be caused by the chronic nature of disease that results in development of specific antibodies against *Trypanosoma* spp. and increasing resistance to concurrent parasitaemia.

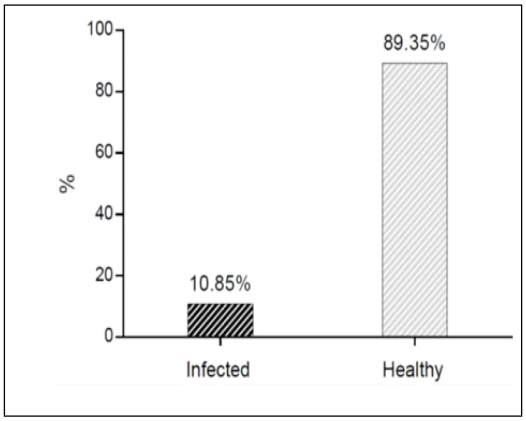


Fig 1: Total molecular results for testing 314 sheep by the conventional PCR assay.

Although, both females and males are grazed out on same pastures, we showed that the parasite is more prevalent in females than males. This might be caused by either the low number of tested males or that females exposed to additional stresses due to pregnancy and lactation.

Hematology

In this study, infected sheep were showed a significant decrease (P<0.05) in values of RBCs and Hb only when compared to those of healthy ones (Table 2). Concerning leukocytes, insignificant differences were seen among infected and healthy sheep (Table 3).

Worldwide, hematology is becoming increasingly more importance in diagnostic and management tools in veterinary medicine. The blood picture of animal provides an opportunity to investigate the presence of abnormalities and help in distinguishing the normal state from diseased one (Roland *et al.,* 2014). Decreased values of total RBCs and

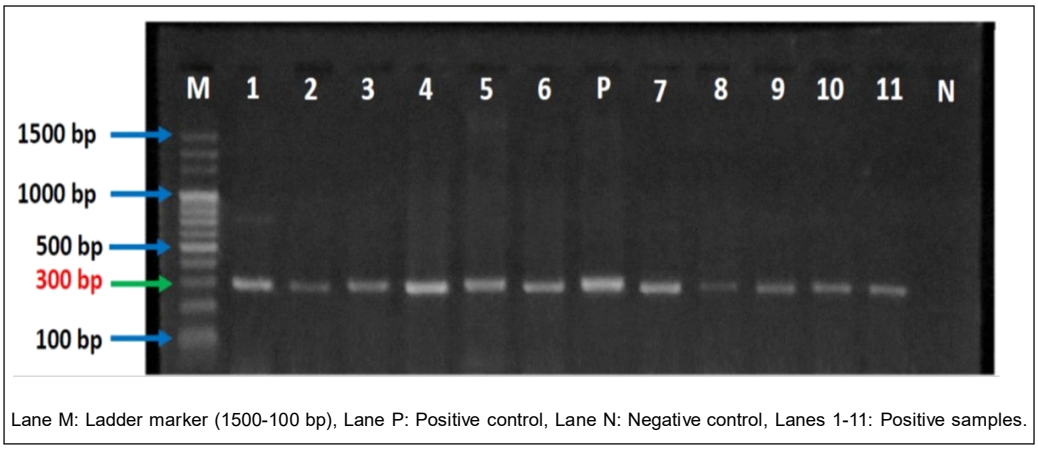


Fig 2: Agarose-gel electrophoresis of some PCR products at 80 Am, 100 Volt for 90 minutes.

Table 1: Association of PCR results to risk factors.

Factor	Group	Total no.	Infected		Odds	Risk
			No.	%		
Sex	Female	227	31	13.66	2.82	2.59
	Male	114	6	5.26	0.35	0.39
	p-value			0.0317*	0.0039**	0.0037**
Age	< 6 months	65	0	0	0	0
	6-12 months	81	3	3.7	0.26	0.26
	> 1 - 3 years	67	15	22.39	3.32	3.32
	> 3 - 4 years	69	11	15.94	2.13	2.13
	> 4 years	59	8	13.56	1.37	1.37
	p-value			0.0328*	0.0048**	0.0057**

Hb of infected sheep with lack of other hematological alteration was seen in this study. Lower hematological values are suggestive an occurrence of anemia that considered the common feature of clinical trypanosomiasis. Boada-Sucre *et al*. (2016) confirmed experimentally that the parasite adhesion to RBCs was decreased significantly at late stage of infection due to the effect of immunity. However, various authors explain that the modification of trypanosomal antigens lead to erythrolysis and removing of infected RBCs (Rossi *et al*., 2017; Rao *et al*., 2021).

Fidelis Junior *et al*. (2016) revealed that leucopenia due to severe neutropenia and lymphopenia were appeared during the acute phase but not at chronic phase in experimentally infected cattle; whereas, Akpan *et al*. (2018) showed that significant leucocytosis due to lymphocytosis and neutrophilia was seen in experimentally infected sheep at 2nd week; however, no significant changes were seen in monocytes, esinophils and basophils. In camel, Derakhshanfar *et al*. (2010) reported that lymphocytosis occurs following chronic infection; while, Sivajothi *et al*. (2015) recognized that leuckocytosis due to lymphocytosis occurs in acute phase and followed by leucopenia during chronic phase.

Biochemical analysis

Data of serum biochemical markers revealed that no significant alteration (P>0.05) was identified in levels of all measured markers except urea that increased significantly (P<0.0356) in infected animals (Table 4). We thought that the type of infection (naturally or experimentally), species of *Trypanosoma* causes the infection and stage of disease have affected largely on the pathogenesis of trypanosomiasis and then physiological disorders occurred. However, the findings of increasing serum urea were

Table 2: Association of PCR results to hematocrit.

Parameter	Unit	Infected	Healthy	p-value
Total RBCs	$\times 10^6$ /µl	9.22±0.26*	9.74±0.12	0.0415 S
PCV	%	31.16±1.06	33±0.74	0.0588 NS
Hb	g/dl	9.38±0.4*	9.9±0.2	0.0418 S
MCV	fl	33.82±0.98	33.89±0.27	0.0807 NS
MCH	pg	10.17±0.31	10.19±0.56	0.094 NS
MCHC	g/dl	30.19±1.48	30.11±1.17	0.099 NS
PLT	$\times 10^3$ /µl	303.33±23.99	347.06±31.54	0.0681 NS

S: Significance * (P<0.05); NS: Non-significance (P>0.05).

Table 3: Association of PCR results to leukocytes.

Parameter	Unit	Infected	Healthy	p-value
Total WBCs	$\times 10^3$ /µl	9.82±0.16	9.5±0.25	0.0542 NS
L	%	42.95±0.84	41.63±0.82	0.0602 NS
N	%	51.26±0.95	52.45±0.96	0.074 NS
L/N	-	1.19±0.03	1.27±0.04	0.0596 NS
M	%	1.55±0.11	1.35±0.19	0.0592 NS
E	%	2.61±0.43	2.34±0.28	0.0541 NS
B	%	0.56±0.07	0.39±0.04	0.058 NS

S: Significance * (P<0.05); NS: Non-significance (P>0.05).

Table 4: Association of PCR results to biochemical markers.

Indices	Unit	Infected	Healthy	p-value
Total protein	g/L	66.45±1.67	67.45±1.69	0.0648 NS
Albumin	g/L	26.71±1.18	27.64±1.16	0.0713 NS
Globulin	g/L	39.82±2.82	39.79±2.82	0.0902 NS
A/G ratio	-	0.69±0.08	0.72±0.08	0.0723 NS
Urea	mmol/L	9.21±0.78*	8.49 0.73	0.0356 S
Creatinine	units/L	86.13±5	93.17±4.72	0.0853 NS
Cholesterol	mmol/L	1.27±0.11	1.45±0.13	0.0656 NS
Glucose	mmol/L	3.26 ±0.28	3.51±0.26	0.0733 NS
Total bilirubin	µmol/L	3.56±0.29	3.12±0.31	0.0597 NS

S: Significance * (P<0.05); NS: Non-significance (P>0.05).

Table 5: Association of PCR results to minerals.

Mineral	Unit	Infected	Healthy	p-value
Ca	mmol/L	2.87±0.088	2.99±0.1	0.0698 NS
Cl	mmol/L	99.76±1.1	100.47±1.2	0.0923 NS
Fe	mmol/L	34.27±2.51	36.43±2.68	0.062 NS
K	mmol/L	4.5±0.28	4.91±0.3	0.0552 NS
Mg	mmol/L	1.06±0.08	1.16±0.05	0.0716 NS
Na	mmol/L	147.24±1.22	148.5±1.36	0.0927 NS
P	mmol/L	1.79±0.1	1.94±0.12	0.0551 NS

S: Significance * (P<0.05); NS: Non-significance (P>0.05).

observed by other studies in monkeys infected with *T. rhoesiense* (Sadun *et al*., 1973), human infected with *T. gambiense* (Awobode, 2006), rabbits infected with *T. congolense* (Ameen *et al*., 2012), sheep infected with *T. vivax* (Onasanya *et al*., 2018), cattle infected with *T. theileri* (Bittner *et al*., 2019) and camels infected with *T. evansi* (Al-Abedi *et al*., 2020). This might because that trypanosome infection in sheep leads to significant pathological and functional disorders in vital organs including liver and kidney.

Mineral status

Regarding values of serum minerals, values of Ca, Cl, Fe, K, Mg, Na and P were showed insignificant alteration in their values when compare between the positively infected sheep with trypanosome and healthy ones (Table 5). Diseases such as trypansomiosis can affect mineral levels in serum of healthy sheep and other domestic animals (Masters, 2018). In Nigeria, Neils *et al*. (2006) detected that serum minerals responded actively in sheep infected experimentally with *T. congolense* suggesting the role of this species in the pathogenesis of disease. Da Silva *et al*. (2011) measured serum minerals in experimentally infected rabbits with *T. evansi* concluding that changes in circulating levels of these markers can be showed when the clinical signs of disease are observed. Hussain *et al*. (2018) mentioned that the pathophysiology or mechanism of trypanosomiosis in naturally infected buffaloes is complicated and multi-factorial in origin, indicating the effect of oxidative products in depletion of minerals. In Germany, Bittner *et al*. (2019) recorded a significant trace mineral deficiency in grazing cattle suggesting the importance of surveillance of mineral status in herds especially when forage quality is low.

CONCLUSION

In Iraq, information concerned the prevalence of *Trypanosoma* spp. in different animals remain very limited in particular in sheep. In this study, molecular PCR assay demonstrated a high efficacy in detection of Trypanosome infections in sheep; however, we recommended to application of phylogeny that can provide more information about the species of parasite and its identity to other global strains. Variation in different hematological and biochemical markers might depend on the *Trypanosoma* species, stage of infection (acute or chronic), type of infection (experimental or natural), species of parasite and animals. Hence, furthermore studies are greatly important to support our information about the prevalence of disease in other areas and in other animals.

Conflict of interest

All authors declare that they have no conflict of interest.

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