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Research Article

Sero-epidemiology, therapeutic study and the risk factors associated with *trypanosomiasis* (Surra) in camels in district Jhang, Punjab, Pakistan

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ABSTRACT

Trypanosomiasis is one of the most pathogenic infections of livestock caused by several *Trypanosoma* species, causing severe economic losses and severe illness by affecting both animals and humans. The main objective of the present study was to determine the seroprevalence and the risk factors associated with *trypanosomiasis* in District Jhang, Punjab, Pakistan. For this purpose, blood samples were randomly collected from 200 camels. The questionnaires were used to collect data on risk factors associated with *trypanosomiasis* before the sample collection. All samples were initially screened by thin smear microscopy and Formol gel test. Later, these samples were further processed by ELISA. The seroprevalence detected by microscopy 22.5% (45/200), Formol-Gel test 37.7% (17/45), and ELISA 44.4% (20/45). The seroprevalence was higher in male camels as compared to females. The variance between the microscopy, the Formol-Gel test and the ELISA test is possibly due to the degradation of antibodies in the spotted samples held at ambient temperature for several weeks. Our study supports the use of antibody detection tests to determine "*Surra*" prevalence in camels rather than parasitological and molecular analysis. This study will help the higher authorities and researchers to take effective control measures against this disease.

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Introduction

Livestock sector is one of the fastest growing sector of the agricultural economy contributing about 11.4% to the Gross Domestic population (GDP) and 53.2% to the agriculture sector (Hussain et al. 2016). Camel is a vital commodity in socio-economic values in Asia and other African countries. In Pakistan, there are about 1.2 million heads of dromedary camel. In particular, the camel is raised for draught purpose, although in some cases they serve human beings by providing milk and meat. Trypanosomiasis (Surra) caused by *trypanosoma evansi* and it is one of the major leading veterinary concern in the world. The ailment has detrimental effects on livestock health resulting in infinite economic losses worldwide (Bhutto et al. 2010).

Trypanosoma evansi (*T. evansi*) is a hemoflagellate that is transmitted by numerous species of blood sucking flies (mostly Tabanids and Stomoxys). However, the vampire bat (*Desmodus rotundus*) serves as a vector and reservoir host in Latin America. In 1880, Griffith Evans initially characterized *T. evansi* using blood samples from horses and dromedary camels (Aregawi et al. 2019). The term "Surra" originated from the Indian language which means "rotten," and it describes the state of animals after the onset of a chronic sickness (Desquesnes et al. 2013). *T. evansi* infects a broad range of animal hosts, with horses, camels, and cattle being the most severely afflicted. In Africa, Asia and the Middle East, it has been diagnosed and represented (Berlin et al. 2012). Surra has negative health consequences that impair production of camel. The sickness has been acknowledged in many areas of Pakistan (Aslam et al. 2010). Multiple species of trypanosomes are found in animals, including *T. bruci, T. vivax, T. evansi, and T. congolense* (Baticados et al. 2011).

Emaciation, infertility, neurological disorder, immunosuppression, anemia, and finally mortality are the clinical manifestations of this illness in both domestic and wild vertebrates (Tehseen et al. 2017). Depending upon the severity of illness, the symptoms often manifest after 15 to 20 days of incubation and include fever, emaciation, anemia, weariness and sloth (Sobia et al. 2018). During the final phase of infection by *T. evansi*, symptoms of nervous system may also arise (Ereqat et al. 2020). Camel and horse populations are especially prone to trypanosomiasis and may expire within a month (Yadav et al. 2014). Simplest way is blood smear microscopy and it is the most often used screening technique for the diagnosis of trypanosomiasis. Thin blood smear slides are stained with Giemsa dye and observed at 100X under light microscope. *T. evansi* is monomorphic, thin parasite possessing an elongating membrane and has a well-developed free follicle outside the cell surface (Muieed et al. 2010). Morphologically, *T. evansi* is a hemi-parasite measuring around 14–29µm in length and 1.5–3.45µm in width and including a nucleus (Hassan-Kadle et al. 2019). However, the incidence of parasitemia is often lower and changes significantly throughout the chronic period; therefore, trypanosoma involvement may go unnoticed.

In order to diagnose camel trypanosomiasis, various serological tests including antibody/antigen detection, enzyme-linked Immunosorbant assay, indirect fluorescent antibody test and card agglutination test detect either *Trypanosoma* antibodies or antigens. Although these tests are sensitive and precise, they cannot distinguish between past and recent infections. For effective therapy and predicting the course of disease, confirmed species detection is necessary. Various chemical agents are being used to tackle trypanosomiasis. Presently, there are six compounds available for the treatment of trypanosomiasis including Isometamedium chloride, melarsomine dihydrochloride, homidium chloride, suramine sodium, quinapyramine sulphate/chloride and diminazene aceturate. Quinapyramine sulphate/chloride and diaminazine aceturate are one of the most frequently used compounds of trypanocide (Fahrimal et al. 2017). Melarsomine dihydrochloride @0.25-0.5 mg/kg body weight is the safest medication for camel against trypanosomiasis. About 60-80% of third world countries depend partially on conventional/herbal medicines which is mainly plants (Maikai et al. 2010). Plants have also been traditionally used for centuries and are now commonly used to cure malaria and other parasitic infections (Omoja et al. 2011). Neem leaves (Azadirachta indica) used traditionally as a medicinal plant for the treatment of viral, bacterial, fungal and trypanosomal infections (Fahrimal et al. 2017). Due to the lack of diagnostic approaches and instruments to quantify the incidence, frequency, and morbidity of the illness in the area, it is impossible to determine the disease's effect on camel in Pakistan. Previous research on the prevalence of trypanosomiasis in horse and bovine has been conducted in Punjab, Sindh, and various parts of Khyber Pakhtunkhwa (KPK) in Pakistan (Hasan et al. 2006) but still the recent literature on prevalence of trypanosomiasis in camel is lacking. Therefore, the current research was conducted to find the incidence of trypanosomiasis in camels in relation to its related risk factors and to evaluate the comparative diagnostic efficiency of biochemical and serological testing and therapeutic trials.

Materials and Methods

Study Area and Source of Samples

The current study was conducted on different breeds of camel in District Jhang (Punjab) Pakistan. Camels to be sampled from various localities were of different age, weight and kept at different farm levels. Two hundred blood samples were taken randomly from camel. Blood samples were obtained from the jugular vein and ear vein of the animals using a 10 ml disposable plastic syringe and an aseptic technique while maintaining the identification of the sampled animal. Five milliliters of blood samples were immediately transferred to purple-capped, spray-coated K2EDTA vacutainer (BD Vacutainer®) tubes. Three milliliters of blood were put in serum separator tubes for serological examination. After 15 minutes of centrifugation at 2000 rpm, the serum was aliquoted and stored at -80°C.

Ethical Approval
The Ethics Committee of the CVAS, Jhang, Punjab, Pakistan originally authorized the current thesis (CVAS/11516, dated 07-01-2020), and later the Directorate of Advanced Studies of the University granted final clearance (DAS/538, dated 15-06-2020). Verbal and written consents were taken from each owner before taking samples of blood and injecting medications to animals.

Microscopic Examination
Blood drop was put on the end of an uncontaminated, dry glass slide. Another dry glass slide with a flat edge was placed at 45°C apart from the blood drop on the first glass slide. When the blood drop was seen spreading along the edges of the spreader glass slide, it was pushed gently to form a good thin smear (Kassa et al. 2011). For each animal two thin blood, slides were prepared. After drying, slides were dipped in methyl alcohol for 3-5 minutes following the procedure as described earlier (Aslam et al. 2010; Berlin et al. 2012). With Giemsa stain prepared smear was stained (also field stain for some samples was used) and observed under oil immersion lens (100X magnification).

Formol-Gel Test
As a screening test, there used formol gel test to detect trypanosomal infection (Aslam et al. 2010). The test was performed as stated previously (OIE, 2012), in smaller tubes Serum samples were briefly transferred and applied two drops of [37 percent formaldehyde (w/v)] concentrated formalin solution. If the sample coagulated and become white suddenly then the study was found positive or if the serum still remained unaffected or coagulated 30 minutes later then negative results will attain.

Serological Analysis
The Enzyme Linked Immunosorbant Assay was performed according the kit's manufacturing instructions. Briefly, 100 ml/well of T. evansi soluble antigen at 5 mg/ml protein concentration was added to Microtest 96-well Polysorp Nunc1 immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 48°C. The plates were blocked with 150 ml/well of blocking buffer, PBS-7% skim milk powder (ref: 190-12865, Wako Pure Chemical Industries' Ltd., Osaka, Japan), with continuous shaking (200 rpm) for 45 min at 37°C. The plates were then rinsed once with phosphate buffer saline (PBS). There was no use for the blocking buffer. The plates were rinsed five times with PBS 0.1% Tween 201 (Sigma-Aldrich) after 30 min in a shaker-incubator at 37°C, 200 rpm (washing buffer, WB). 100ml of peroxidase-conjugated anti-bovine IgG (A5295, Sigma–Aldrich), a 1:10,000 dilution in blocking buffer of peroxidase-conjugated anti-bovine IgG (A5295, Sigma-Aldrich) was added, and the plates were incubated for 30 min at 37°C with constant shaking (200 rpm). 100ml of the complex substrate/chromogen 3,30,5,50-tetramethylbenzidine (TMB) (K blue1 TMB substrate, Neogen Europe Ltd., Scotland, UK) was added after five washes with washing buffer. The plates were shaken and then incubated in a dark room for 30 min. Optical density (OD) was measured at 630 nm in an ELISA reader (Dynex Technologies1, VA, USA), (Tehseen et al. 2017).

Therapeutic Trials
Total number of 18 positive sample were randomly separated into groups A, B, C for treatment trials against *T. evansi*. Different therapeutic agents were delivered to each group to determine the comparative effectiveness of medications. Group A camels were administered Tryban® (quinapyramine sulphate) at 5 mg/kg body weight IM or SC (UM Enterprises). Group C camels were administered IM doses of 3.5-7 mg/kg of Diminazene® (Diminazene diaceturate) for three days (Star Laboratories Pvt. Ltd.). Group C was treated against trypanosomiasis with powdered Neem leaves (Azadirachta indica) at a dose of 60 g orally each day.

Re-Examination of Treated Animals
After elapsing 7 days' post-treatment, blood samples were taken from previously positive animals to check the status of the disappearance of piroplasm through Giemsa stained thin blood smears examination.

Therapeutic Efficacy of Drugs
The formula was applied to check the efficacy of used drugs.
Therapeutic efficacy Percentage = No. of recovered animals **/** No. of total animals × 100

Statistical analysis
The factors relevant to the prevalence of the protozoan were examined using the Pearson Chi-square test, while the therapeutic efficiency of the chosen medications was calculated using SPSS version 24.

Results
A total of 200 camels were screened for *T. evansi* infection. Out of 200 blood samples tested, 45 (22.5%) were detected positive through microscopy. These positive samples (n = 45) were further analyzed using the Formol-Gel test and ELISA. The overall prevalence was recorded as 22.5 % by microscopy, 37.7% (17/45) by Formol gel test and 44.4% (20/45) by ELISA as shown in Table 1. The result showed that the prevalence was significantly higher in the age group >4 years (28%) than the 1-4 years' age group (17.1%) and <1 years (16.6%). Prevalence was higher in male camels (25%) than female camels (18.7 %). The lower prevalence in camel for breeding purpose at the farm level (16.6%) can be linked to good management and accessibility of veterinary practitioners. Camels with poor body condition score (BCS) and used for draught purpose at brick-kilns showed higher prevalence (51.4%) for *T. evansi* due to stress, over workload, poor nutrition and diet, and, hence other infections as well. In the perspective of insects control environment, it was evident that the camels found with insects controlled environment were recorded with significantly lower infection than the animals with poor insects controlled environment. Sex, age, and body condition were factors significantly associated with seroprevalence of diseases ($P < 0.05$) whereas the other factors were not ($p > 0.05$) as shown in Table 2.

Table 1: Prevalence of *trypanosomiasis* in camels detected by using different diagnostic techniques

Test	Total No. of samples	Positive samples	
		No	%
Thin Blood smear microscopy	200	45	22.5%
Formol-gel Test	45	17	37.7%
ELISA	45	20	44.4%

Table 2: Prevalence of *Trypanosoma evansi* and its associated risk factors in camels based on microscopic examination

Variables	Level	No. of Animals tested	Positive	Prevalence %	*p*-value
Sex	Male	120	30	25	0.011
	Female	80	15	18.7	
Age	>4 years	100	28	28	0.032
	1-4 years	70	12	17.1	
	< 1 year	30	5	16.6	
Purpose	Draught	140	35	25	0.027
	Breeding	60	10	16.6	
Body Condition	Good	130	9	6.9	0.012
	Emaciated	70	36	51.4	
Insects control	No	140	34	24.2	0.25
	Yes	60	11	18.3	

Table 3: Drug trials and their efficacy percentage

Group	Therapeutic agents and their composition Dosage and route of administration	No. of animals	No. of animals recovered	Drug efficacy
A	Tryban® (Quinapyramine methyl sulphate) @ 5mg/kg body weight SC (UM Enterprises)	6	6	100%
B	Diminazene® (diminazene diaceturate) @ 3.5-7mg/kg IM for 3 days (Star laboratories Pvt. Ltd)	6	4	66.6%
C	Neem leaves powder (Azadirachta Indica) @ 60g daily PO for 7 days	6	3	50%

In group A, treated with Tryban® (quinapyramine sulphate) 5 mg/kg SC and supportive therapy all the six animal were recovered and showed 100% cure rate. The group B administered with 3.7–7.5 mg/kg IM of Diminazene aceturate, four animals out of six animals were recovered. Diminazene aceturate was 66.6% effective. Group C administered with 60 g of powdered Neem leaves (*Azadirachta indica*) orally every day for seven days along with supportive therapy. Three out of six animals were recovered with no adverse consequences. Neem leaf powder (*Azadirachta indica*) was 50% effective. The medicine Quinapyramine sulphate and powdered Neem leaves (*Azadirachta indica*) were very successful in eliminating trypanosomes from the blood with no adverse effects.

Discussion

Trypanosoma commonly known as 'Surra' a protozoa parasite of mammalian blood, induces a disease in various animals such as buffalo, cattle, camel, and horse. The clinical indications of the *trypanosomiasis* by *T. evansi* can be acute or chronic. In certain cases, the acute symptoms include fever, anemia, emaciation, and sudden death of the animal. In affected animals, the infection reveals typical signs such as fever, emaciation, anemia, fatigue, conjunctivitis and lymphadenomegaly (Sobia et al. 2018).

The current study was conducted with 384 blood samples collected from horses and camels from District Jhang, Punjab, Pakistan. Sampling was done through different locations in and around the Jhang. To endorse whether animal was sick or not, various tests were used including formol gel (FG) test, thin blood smears microscopy, and PCR. Microscopy and PCR were regarded as specific tests for the detection of Trypanosoma species, while FG was used to confirm whether the animal was sick or not. various

studies support the use of these tests particularly against parasite like Trypanosoma species (Muieed et al. 2010).

In the current study prevalence of *trypanosomiasis* by microscopically examination from camels was 22.5%. From Sindh Pakistan same tests were applied to detect *trypanosomiasis* in camels from Sindh, Pakistan and the overall prevalence was 13.7 percent**.** The higher infection rate of *trypanosomiasis* in Sindh, Pakistan is due to difference in ecological circumstances and climate changes than Punjab. This technique has also been used for detection of *trypanosome evansi* prevalence in equines from Punjab, Pakistan (Hasan et al. 2006). Similarly, from Gujranwala, Pakistan 2% prevalence was reported in horses and donkeys by thin smear blood microscopy (Aslam *et al*, 2010). The effectual use of microscopy in the diagnosis of *Trypanosoma spp*. has been reported by a number of international studies. From Iraq, it reported 56.25% prevalence of hemoparasites in camels (Al-Amery et al. 2017) from Iran 17.61% (Mirshekar et al. 2017) and from Egypt 0.96%.

The Formol-gel test is a common biochemical test used to determine infection in chronic stage. However, researchers have tried to report the prevalence of Trypanosoma spp. such as Sobia et al. (2018) in Pakistan, where the ort formol gel test indicated a prevalence of 51.1% in cattle, goats, sheep, donkeys, and camels. Similarly, 120 out of 500 (24.0%) horses in Lahore, Pakistan, tested positive for the presence of formol gel (Aslam et al. 2010; Tehseen et al. 2017). According to the current research, ELISA-based identification procedures are more accurate than microscopy and other methods (Desquesnes et al. 2013). In addition, large-scale examinations of trypanosome samples used this technique (Tran et al. 2009). (Adel et al. 2019) discovered that ELISA Polysorp Nunc1 was more specific for the detection of trypanosomal antigens. The results of the present study were comparable to those of Adel et al. (2019). In separate research (El Wathing et al. 2016), it was shown that the use of PCR in the diagnosis of trypanosomiasis is more sensitive and accurate, especially in instances with low infection rates, as well as potentially useful for epidemiological investigations. Similarly, Elbalkemy et al. (2016) discovered that the ELISA test sensitivity for detecting trypanosomes in blood samples from horses and donkeys was greater than the Formol Gel test sensitivity. In India, quinapyramine sulphate has also been used to treat T. evansi (Hota et al. 2019). Additionally, quinapyramine has been used to treat "Surra" in horses and camels (Matovu et al. 2020). The dimenazine acciturate was shown to be effective against the illness to the extent of 66.6%; however, as previously reported, it caused unpleasant reactions (Raftery et al. 2018). Group C administered with Neem Leaves powder (*Azadirachta Indica*) has shown to be 50% effective in healing animals with fewer hazardous side effects. These outcomes parallel those of the research published by Omoja et al. (2011). The results of Mahboob et al. (2008), who administered the same amount of dried Azadirachta indica leaves to horses afflicted with strongylosis, are consistent with the present findings. It indicates the efficacy of neem leave in multiple diseases. (Amin et al. 2011) reported the efficiency of 10 percent neem water extract against nematodes in sheep, as well as substantial decreases in EPG count. *Azadirachta indica's* anti-trypanosomal effectiveness may be attributed to the presence of secondary metabolites capable of creating radicals that work against the parasite's metabolism (Martina Enyanwu, 2018). Yadav (2014) evaluated the effectiveness of leaves powder (*Azadirachata indica*) at a dosage rate of 525 mg/kg body weight against the parasitic gastrointestinal nematode.

Trypanosomes' pathogenic impact is also induced by the secretion of cytokines and nitric oxide. Active Neem (*Azadirachta indica*) components such as phenol neutralize toxic substances, thereby extending the organism's life cycle. Low amounts of active chemicals in the aqueous extract may account for the extract's inability to eradicate the parasite fully, as stated by Ajabe et al. (2018).

Conclusion

It was concluded that subacute and chronic Trypanosomiasis "Surra" in camel is widely present in Jhang, Punjab Pakistan. The seroprevalence was higher in male camels as compared to females. The ELISA test revealed more instances of trypanosomal infection in camels than the Formol Gel test and the thin blood smear microscopy, demonstrating its diagnostic superiority. The quinapyramine should be employed for treatment of positive animals. Neem leaf powder (*Azadirachta indica*) contains anti-trypanosomal properties which can be used as alternative therapy in the treatment of camel trypanosomiasis. This study suggested that higher authorities and government should make policy to control this disease.

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Author Contribution: All authors contributed to the study conception, design and analysis.

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