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Clinical phases of camels *surra* regarding cytokines genes quantitative expression as analytical biomarkers of infection

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In the present investigation, it was intended to characterize the clinical phases of trypanosomiasis in infected camels, in relation to difference in regulation of cytokines expression. The diagnostic potential of IL-4, IL-6, IL-8, and IL-10 as biomarkers of clinical phase of infections in comparison to other technologies including parasitological, serological, as well as conventional PCR to improve laboratory and/or field diagnosis was studied. The study included 11 localities in Egyptian desert included: North West Coast, Western Desert Oasis, South East Coast, Suez Canal region and Sinai. Blood specimens were collected from 110 camels examined seasonally (4 times/year) from June 2014 to July 2016. *trypanozoon* parasites were detect in blood by wet blood smears (6.7%), field stained blood film (7.72%), and hematocrit centrifugation (14.66%) within investigated localities in infected camels with high parasitaemia (≥ 40 trypanosome/field) and acute infection. On the other hand, serological diagnosis by ELISA; using homemade *trypanozoon* lysed antigen, as well as, CATT test revealed 44.1% and 41.36% seropositive incidence for *surra*, respectively. On the other hands, molecular screening for trypanosomiasis was more specific and sensitive. The *tryp*1 primers pair specifically amplify intragenic spacer I region (*its*1) of ribosomal DNA of all *Trypanosomes*; therefore, was the primary target during PCR diagnosis. While, the *tbr* primers pair could amplify satellite genomic DNA of *trypanozoon* parasites; thus used as the confirmatory second choice. PCR screening of camels samples utilizing the specific primers of ITSI and SND, revealed 68.3% overall positivity, while seasonal incidence recorded 7.73%, 19.20%, 33.3%, and 8.07% during autumn, spring, summer, and winter, respectively. Positive PCRs were then typed into species/sub-genus level by sequencing then alignment into GenBank database. The annotated fragments revealed infections with *T. evansi*; the only species of *Trypanozoon* sub-genus possibly present in Egypt. Sequences alignments show clustering based on the biological origin of the isolates; camel breed local or imported with tendency for clonality that was geographical-dependent. The influence of *Trypanosoma evansi* on cytokines mRNAs expression of IL-4, IL-6, IL-8 and IL-10 in blood cells were quantified, and then normalized with housekeeping gene (β-Actin), finally expressed as fold of induction. The qRT-PCR revealed up regulation then down regulation of target cytokine genes in response to acute (high parasitemia) then sub-acute but not with chronic infections, respectively. Despite that sub-acute infected individuals showed fold increase in cytokines genes

expression, but were lower than those recorded for acute infections. However, the fold increase was higher for IL-8 and IL-10 than those recorded for IL-4 and IL-6, calculated 11.69 ± 4.146 and 13.04 ± 7.103 for IL-8 and IL-10, respectively (Means St. Dev. 2^{-} ($\Delta\Delta$CT). In conclusion: The results of this study indicate that camels' isolates of *T. evansi* belong to homologous phylogroup/serogroups/clade that contained a variety of virulence associated traits. On the other hand, the investigation in rodents needs to be completed to evaluate their role as reservoirs of potentially pathogenic trypanosomes in the vicinity of humans and livestock. Finally, a wide range of genes played role in the efficiency of immune reaction associated with *surra*, however, their inclusion was beyond the scope of the current study.

Keywords: *Trypanosoma evansi*, Intragenic Spacer I, Satellite Genomic DNA, Parasitemia, Chemokines, qRT-PCR.

INTRODUCTION

The word "*surra*" is Indian meant "rotten" which described the state of the camel after chronic phase of the infection (Vittoz, 1995). *Trypanosoma evansi* is the main causative agent of animal trypanosomiasis; *surra* (Amoudi et al., 2011; Haridy et al., 2011). Clinical infections are found under more than 30 vernacular names (Hoare, 1972; Stephen, 1986; Atarhouch et al., 2003; Antoine-Moussiaux et al., 2007). The incidence of infections found increasing significantly during the season when biting fly populations were greatly increased. Not all mammalian hosts were found equally susceptible to infection, hence, the disease severity is species dependent (Zayed et al., 2010a and 2010b). *Surra* affecting mainly camels and horses, yet buffaloes and cattle were also affected (Milocco et al., 2013). Additional highly at risk species included; donkeys, mules, deer, llamas, dogs, and cats. The endemic status of *T. evansi* in dromedary camels is well documented in Egypt (Abdel-Latif, 1957; Abdel-Ghaffar, 1960; El-Sawalhy and Ebid, 1994; El-Said et al., 1998; El-Said, 1999; Zayed et al., 2010a &2010b) but information about infectivity in hosts other than camels is still lacking (Hilali et al., 1998 & 2004; Zayed et al., 2010a & 2010b).

Trypanosomes are flagellated protozoa of the family *Trypanosomatidae*, genus *Trypanosoma*, subgenus *Trypanozoon* which is separated into two subdivisions according to site of biological development in the digestive tract of the vector; *salivarian* (anterior) are pathogenic, and *stercorarian* (posterior) are non-pathogenic trypanosomes, except *T. cruzi*. They could be transmitted either directly; transplacental, oral, venereal, and mechanical by insect bite, or indirect biological transmission by vampire bats and vectors (Hoare, 1972; Hilali et al., 1998 & 2004; Zayed et al., 2010a & 2010b). *T. brucei brucei* was thought to be the ancestral origin of *T. evansi;* after losing the maxicircles of kinetoplastic mitochondrial DNA, therefore, no longer was able to undergo its cycle in Glossina; insect vector (Borst *et al.,* 1987). It was previously reported that such genetic alteration had occurred in camels (*Camelus dromedarius*), the preferred host species where *T. evansi* early developed from *T. brucei brucei* (Lai et al., 2008).

In the epidemiological viewpoint, studying the patterns of susceptibility to infection and the clinical phase of disease caused by *T. evansi* in the camels might help to identify mechanisms that lead to the design of new control measures (Hilali et al., 1998 & 2004; Zayed et al., 2010a & 2010b; Amer et al., 2011; Salim et al., 2011). Due to the absence of specific pathognomonic signs, clinical examination is useless in the differential diagnosis of animal trypanosomosis (Losos, 1986; Desquesnes, 1997; Desquesnes et al., 2007). Definitive diagnosis of the infection was based on the trypanosomes identification in blood samples by parasitological techniques (Hilali et al., 1998 & 2004; Zayed et al., 2010a & 2010b). Recently, DNA technology had remarkably improved *trypanosomes* detection and/or characterization without all biological hazardous associated the traditional parasitological techniques. Therefore, PCR was recommended for its consistence and sensitivity than parasitological techniques, especially in antigenaemic yet aparasitaemic host animals (Majiwa et al., 1994; Desquesnes and Tresse, 1996; Almeida et al., 1997; Desquesnes, 1997; Desquesnes and Dávila, 2002; Milocco et al., 2013).

Understanding the onset, intensity, and thresholds of pathogen count that triggering regulatory cascades of the immune response during disease could be associated clinical stage as biomarker of the infection (Leutenegger et al., 2000; Almeida et al., 2011; Hogeveen et al., 2011; Günther et al., 2016; Hakim et al., 2017; Allam et al., 2017 and 2018). Nevertheless, it is important for the design of diagnostics, prophylactic vaccines, and for optimization of therapeutic protocols (Brown, et al., 1990; El-Amin et al., 1998Günther et al., 2016; Hakim et al., 2017; Allam et al., 2017 and 2018). Due to, the

mammals' immune cascades are designated to respond hastily to pathogen intrusion through the activation of numerous pattern recognition receptors (PRR) (Goldammer et al., 2004; Ibrahim et al., 2012; Gilbert et al., 2013). Therefore, up regulation of Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4), Tumor Necrosis Factor-α (TNF-α), Interleukin-1α (IL-1α), IL-4, IL-6, IL-8, IL-10 and the NFκB pathway activation (Leutenegger et al., 2000; Almeida et al., 2011; Günther et al., 2016; Hakim et al., 2017; Allam et al., 2017 & 2018). Frequently, this activation is able to clear most of the infections despite their inducer type. Nevertheless, pathogens had persistence and/or recurrence behavior lead to unrecoverable infections. *T. evansi* had diverse incidence rates and clinical phase of infection (Majiwa et al., 1994; Desquesnes and Tresse, 1996; Almeida et al., 1997; Desquesnes, 1997; Desquesnes and Dávila, 2002; Zayed et al., 2010b; Milocco et al., 2013).

In camels, most internal infections induce limited clinical signs and/or frequent eradication. On the other hand, systemic infections provoke dynamic motivation of immune system characterized by different regulation of chemokines and cytokines, leading to stimulation and/or inhibition of the local and generalized immune response of camels (Almeida et al., 2011; Hogeveen et al., 2011; Allam et al., 2017; Günther et al., 2016).

In the present investigation, it was intended to characterize the clinical phases of *T. evansi* in infected camels, in relation to difference in regulation of cytokines excepression. The diagnostic potential of IL-4, IL-6, IL-8, and IL-10 as biomarkers of clinical phase of infections in comparison to other technologies including parasitological, serological, as well as conventional PCR to improve laboratory and/or field diagnosis was studied.

MATERIALS AND METHODS

Geographical Scope of the Study:

Sampling localities in eight provinces from Egypt were selected in the study (table 1).

Ethical Approval:

All procedures were in accordance with the ARRIVE guidelines which were in accordance to the European (EU) Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023 revised 1978). In addition, were in agreement with the adopted ethics guidelines of the ministry of higher education and scientific research, national research center and desert research center for the care and use of animals in Egypt.

Camels Blood Samples:

A total number of 110 camels (*Camelus dromedarius*) were investigated for *Surra*, which were examined then sampled seasonally (4 times/year) during the study period from June 2014 to July 2016. EDTA-whole blood samples were collected from jugular veins and used for camels' hematological parameters estimation, trypanozoon separation and parasitological diagnosis of infection. RNA later (Sigma-Aldrich) was added to each blood samples after collection, then the remaining were stored at -80 °C until RNA & DNA was extracted for molecular studies (Allam et al., 2018). Additional 10 ml blood/host/trip was collected on plan tubes for serum separation after centrifugation at 2000 rpm for 10 min then stored at -20 °C until used during sero-diagnosis and camels biochemical parameters estimation.

Camels Medical Assessment regarding Hematological and Biochemical Profiles:

Routine medical inspection of camels' population was intended for external parasites detection and/or treatment, where each camel had its epidemiological records which was designed previously (Yassin et al., 2016-2017; Allam et al., 2018). Hematological parameters investigated were; total erythrocyte count (RBCs), total leucocyte count (WBCs), differential leucocyte count (DLC), hemoglobin (Hb) and packed cell volume (PCV) done as described by Schalm et al., (1986). Serum biochemical parameters which were determined spectrophotometerically included; total protein (Biuret method), albumin, globulin, albumin/globulin ratio, AST, ALT, ALP, Creatinine, and Urea was determined according to previous protocols (Schirmeister, 1940; Gornal et al., 1949; Reitman and Frankel, 1957; Fawcett and Soctt, 1960; Belfield and Goldberg, 1971; Doumas et al., 1971). Statistical analysis for hematological and biochemical parameters was performed using Student's *t* test (SPSS 14.0 for Windows Evaluation Version). Probability values (*P*-value) $\leq$ 0.05 were considered of statistical significant and $\leq$ 0.001 were considered of high statistical significant (Alan and Duncan, 2011).

Table (1): The coordinates of the regions and localities of the Egyptian deserts studied.

Region/Province	City/Locality	Coordinates
North West Coast	Mariout	31° 9′ 11″ North, 29° 53′ 55″ East
	Marsa Matruh	31° 21′ 0″ North, 27° 14′ 0″ East
	El Salloum	31° 30′ 13″ North, 25° 6′ 54″ East
Ismailia	Ismailia	30° 36′ 0″ North, 32° 17′ 0″ East
Southern Sinai	Wadi Gharandal	29° 30′ 0″ North, 33° 50′ 0″ East
Middle Sinai	Al hasna	30° 30′ 0″ North, 33° 36′ 0″ East
Northern Sinai	Bir Al abed	31° 0' 59" North, 33° 0' 38" East
Western Desert	Siwa Oasis	29° 11′ 0″ North, 25° 33′ 0″ East
New Valley	Dakhla Oasis	25° 29′ 29.6″ North, 28° 58′ 45.2″ East
South East Coast	Shalateen	23° 7′ 54″ North, 35° 35′ 8″ East

Parasitological Diagnosis:

Wet Blood Smears Examination:

Was carried out by light microscope according to procedures described by Hoare (Hoare, 1972) using the morphological characteristics of *trypanosomes*.

Stained Blood Smears Examination:

Two blood films were prepared from each sample, then air dried at room temperature. The films were fixed with methyl alcohol for 5 minutes, left to dry then stained with Field's stain as previously described (Fleck and Moody, 1993), finally examined by light microscope under oil immersion lens.

Hematocrit Centrifugation for *Trypanosomes* Concentration and Separation:

In cases of low parasitaemia (≤ 40 trypanosome/field [10^7 trypanosomes]), infection could not be detected by wet nor stained blood films examination (El-Sawalhy and Ebeid, 1994; Taylor et al., 2008; Zayed et al., 2010a). Two hematocrit centrifuge tubes from each blood sample were centrifuged at 12000 rpm for 4 minutes. Then tubes were examined under oil immersion lens at junction of plasma and buffy coats to detect the *trypanosomes* motility. To confirm the diagnosis, stained blood films were prepared and then examined.

Serological Diagnosis:

Whole Cell Lysate of *T. Evansi* Field Isolates from Camels:

Whole-cell lysate (WCL) antigens were prepared according to a standard method described previously (Giardina et al., 2004; Zayed et al., 2010a & 2010b). During this procedure, hematocrit centrifugation isolation was done for *T. evansi* from camels' blood, then *trypanosomes* were collected from buffy coat layer, and erythrocyte were lysed (Murray et al., 1977). The collected pellets were then suspended separately in electrophoresis sample buffer (50 mM Tris-HCL, pH 7.5, 2.0% SDS, 2.0% β-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue, Sigma Aldrich) boiled for 5 min then cooled on ice (Zayed et al., 2010a & 2010b). The supernatant of lysate trypanosomes were collected separately then concentrated in dialysis bags with polyethylene glycol and used as WCL antigens. The total protein content of the concentrated antigens was estimated by the procedure described previously by Lowery et al., (1951).

Indirect Solid Phase Enzyme Linked Immunosorbent Assay (ELISA):

The optimal antigen concentration was first adjusted to be 30µg/ml during check board titration (Zayed et al., 2010b). ELISA was applied on camels' sera at dilution 1:100 against the prepared *T. evansi* antigens (Zayed et al., 2010b). All antigens and control sera were diluted in carbonate-bicarbonate coating buffer to 1:10 (Zayed et al., 2010b). Absorbencies were read using the automated ELISA reader at a wave lengths 450 nm and 405 nm, then cut off values were calculated (Lejon et al., 2003).

Card Agglutination Test (CATT):

In each drawn well on the supplied test card with the kit, 25µl of diluted sera were added, and then 45µl of well-homogenized CATT antigen was added to each well. The mixture was thoroughly mixed and spread out to about 1mm diameter using a stirring rod. The card was agitated in a circular motion using electric rotator at 60-70 rpm/ 5 min at room temperature (Zayed et al., 2010b). Samples developed blue granular agglutination

was considered positive, which were read in comparison with the control wells and according to supplied instructions (Laha and Sasmal, 2008).

Statistical analysis:

The seasonal incidence of *Trypanosoma* and the methods of applied technique were statistically analyzed by using Model GLM of SAS software version (9.0) (SAS 2009). Duncan Multiple Range test was used to test the level of significance among the means (Snedecor and Cochran, 1989).

Molecular Diagnosis of *Surra*:

DNA Purification:

Genomic DNA was extracted from camel blood specimens using GF-1 Tissue Blood Combi DNA Extraction Kit (SNF, Vivantis, Malaysia) according to the manufacturer's instructions. DNA concentration were measured by Nanodrop 2000c (Thermo Scientific), adjusted to 100 ng/µl genomic DNA working concentration.

Detection and Characterization of *T. evansi* by Multi-Genes Amplification then Sequencing:

The amplification reactions during the present study were performed in a PTC-100™ thermal cycler (MJ Research Inc., USA) under complete aseptic conditions according to previous publications (Masiga et al., 1992; Desquesnes et al., 2002). Each 25 µl total volumes of a PCR mixture contained 25-50 ng/µl camel genomic DNA, 10 pM/µl of each primers, 12.5 µl of 2x Dream Taq Green PCR master mix (Thermo Scientific) and 9 µl nuclease free water (Qiagen) to complete the total volume of the reactions. A reagent blank was run simultaneously as negative control with each PCR. The primers' sets were designed according to previous publications (Masiga et al., 1992; Desquesnes et al., 2002), with predicted products as illustrated in table (2), respectively. Amplified products with regards to cycling profile in table (3) were electrophoresed in 1% agarose gels in TBE buffer then stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Alliance Bio, USA) was used with each gel. Gels photos were analyzed by Lab Image software (BioRad). Sequencing reactions were performed in an MJ Research PTC-225 Peltier thermal cycler using an ABI PRISM®BigDye™ Terminator cycle sequencing kits with AmpliTaq® DNA polymerase (Applied Biosystems), following the protocols supplied by the manufacturer.

Internal Quality Control of PCRs:

A semi-qualitative internal control to verify the efficiencies of DNA isolation and PCR assays were applied (Konnai et al., 2006). The primer used derived from highly conserved regions in the host species genomes; β-globin gene sequences (Table 2). All amplifications were performed in a PTC-100™ thermal cycler (MJ Research) under previously publicized amplification conditions (Konnai et al., 2006), table (3).

Data analyses by NCBI blasten:

The obtained sequences were aligned, assembled and corrected using Chromas Pro 1.49 beta (Technelysium) and Benedict sequence alignment editor (v. 7.0.9.0). Each fragment's sequence was analyzed using Blastn program in NCBI interface (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence homology searches against *Trypanosomes* GenBank database. Multiple sequences alignments for evolutionary relationship in between new Egyptian isolate and Genbank reference isolates were inferred (Regnery et al., 1991; Tamura et al., 2004).

Quantitation of Cytokines Expression by qRT-PCR:

RNA Extraction and Quality Assessment:

Total RNA was extracted by a double extraction method first using Trizol (Invitrogen) and then RNeasy (Qiagen) column purification according to manufactures instructions. RNA integrity, purity and quantity determination were estimated using Nanodrop 2000 (Thermo scientific). The residual genomic DNA was removed by DNA digestion with RNase-free DNase-I (Qiagen) at 37 °C for 10 min, then heat inactivated at 95 °C for 5 min, finally chilled on ice (Sambrook et al., 1989).

RT Assays:

Reverse transcription of total mRNAs into cDNAs copies were carried out as illustrated in table (3) using 1 µg of each mRNA obtained sample, incubated with 1 µg of random primers (Promega) for 10 min at 65 °C, and then for 5 min on ice in a final volume of 10 µl as hot start reaction. Reverse transcription mixture contained 15 U of avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Promega), AMV RT buffer (Promega), 4 mM deoxynucleoside triphosphate (dNTP) (Promega), and 40 U of RNasin (Promega)

Table (2): Synthesized oligonucleotides primers used during this study.

Primers	Targets	5`-Sequence-3`	Fragments Length	References
Internal quality control of PCR				
β-glob-F **β-glob-R**	Host β-globin gene	5`-ACACAACTGTGTTCACTAGC-3` 5`-CAACTTCATCCACGTTCACC-3`	350	Konnai et al., (2006)
***Trypanozoon* Genomes**				
TRYP1-F **TRYP1-R**	Ribosomal Intragenic Spacer Region I (ITS1)	5`-CGTCCCTGCCATTTGTACACAC-3` 5`- GGAAGCCAAGTCATCCATCG -3`	520-630	Desquesnes et al., (2002)
TBR1-F **TBR2-R**	Satellite Nuclear DNA (SND)	5`- GAATATTAAACAATGCGCAG -3` 5`- CCATTTATTAGCTTTGTTGC -3`	164	Masiga et al., (1992)
Cytokines Genes				
IL4-F IL4-R	Interleukin-4	5`-CATGCATGGAGCTGCCTGTA-3` 5`-AATTCCAACCCTGCAGAAGGT-3`	41	Leutenegger et al., (2000) Moussay et al., (2006)
IL6-F **IL6-R**	Interleukin-6	5`- TCATTAAGCGCATGGTCGACAAA-3` 5`- TCAGCTTATTTTCTGCCAGTGTCT-3`	47	
IL8-F **IL8-R**	Interleukin-8	5`- CACTGTGAAAATTCAGAAATCATTGTTA-3` 5`- CTTCACAAATACCTGCACAACCTTC-3`	53	
IL10-F **IL10-R**	Interleukin-10	5`-CCAAGCCTTGTCGGAAATGA-3` 5`-GTTCACGTGCTCCTTGATGTCA-3`	42	
Internal Quality Control of qRT-PCR				
βact-F **βact-R**	β-actin	5`-CCTTTTACAACGAGCTGCGTGTG-3` 5`-ACGTAGCAGAGCTTCTCCTTGATG-3`	47	Hoorfar et al., (2004)

Table (3): The Thermo-cycling profiles applied during PCRs and qRT-PCR.

Genomes	***T. evansi***		**Camels**					
Techniques / **& Genes Steps**	**PCRs**		**PCR**		**RT**		**qPCR**	
	ITS1 & SND	**Cycles**	**β-globin Gene**	**Cycles**	**Random Primers**	**Cycles**	**β-actin & Cytokines Genes**	**Cycles**
Initial Step	94 °C/ 3 min	1	94 °C/ 3 min	1	65 °C/ 10 min Ice/ 5 min	1	95 °C/ 10 min	1
Denaturation	94 °C/ 1 min	30	94 °C/ 1 min	30			95 °C/30 sec	40
Annealing	60 °C/ 1.5 min		60 °C/ 1.5 min		42 °C /1.5 h	1	58-65 °C/30 sec	
Extension	72 °C/ 2 min		72 °C/ 2 min				72 °C/ 30 sec	
Final Step	72 °C/ 10 min 4 min/ till stored	1	72 °C/ 10 min 4 min/ till stored	1	95 °C/ 5 min 4 min/ till stored	1	72 °C/ 10 min 4 min/ till stored	1

Diluted cDNA samples were stored at 4 °C until used in qPCR reactions, then kept at -80 °C.

Relative Quantification of Cytokines by qPCR Assays:

The primers of IL-4, IL-6, IL-8 and IL-10 genes along with β-actin gene housekeeping gene (Table 2) utilized in this study were designed using publicly available cytokines genes sequences then were purchased (Qiagen). In addition, Quantitect probe designed for RT PCR (Qiagen) was also used according to manufacturer. Primers design considered to span an intron-exon boundary of target genes to prevent the amplification of genomic DNA (Leutenegger et al., 2000; Moussay et al., 2006; Allam et al., 2018). The reaction condition for each individual gene was optimized using Quantitect SYBR green PCR kit (Qiagen) then amplified in Rotor-Gene (Qiagen). The amplification was carried out in a final reaction volume of 25 µl. The qPCR protocol designed according to cycling conditions illustrated in table (3) (Bougarn et al., 2010; Allam et al., 2018). The fluorescence signals were measured once at the end of extension step/cycle/gene. For each sample a dissociation curve was generated after completion of amplification and was analyzed to determine specificity of qPCR reaction. The normalization factors NFn and NFn+1 were calculated. Values for target genes were normalized by the internal positive control (β-actin). Relative transcript quantification standard curves were plotted using a six fold serial dilution of cDNA (Livak and Schmittgen, 2001). The relative level of expression for gene of interest was analyzed using the delta-Ct method ($2^{-\triangle\triangle Ct}$) which was normalized by dividing by a proper normalization factor; $2^{-\triangle\triangle Ct} > 1$ for up regulation, $2^{-\triangle\triangle Ct} < 1$ down regulation, and $2^{-\triangle\triangle Ct} = 1$ no change in gene expression (Livak and Schmittgen, 2001; Allam et al., 2017 & 2018). Analysis of the melting curve of specific qPCR products was performed by slowly raising the temperature from 60 °C to 95 °C by means of regular fluorescence measurements, which should be distinguished from primer dimmers (dissociation temperature < 74 °C) (Alan and Duncan, 2011; Allam et al., 2017 & 2018).

RESULTS

Medical Inspection and Hematological and biochemical Profiles of Investigated Camels' Population:

The epidemiological records of each camel included full data about; age, sex, breed, the purpose of rearing animal, production/reproduction records, external parasites infestation, date of samples collection, clinical signs, suspected disease, and time and type of any treatments. The main clinical signs observed on infested camels during sampling were fever, anorexia, lethargy, anemia, enlargement of superficial lymph nodes and emaciation, other than being apparently healthy in the majority of the inspected population. Older individuals were more susceptible for external parasites infestation; it was more prevalent in males.

The hematological and biochemical parameters were estimated for all camels' population, simultaneously, during each sampling trip. The results revealed macrocytic anemia and leucopenia recorded in infected camels; moreover, advanced normochromic anemia was more common in females than males. However, there were no significant differences in biochemical changes between infected and treated camels. The biochemical and hematological parameters for investigated camels representing health status during infection and after treatment are illustrated in Table (4) and Charts (1), respectively.

Morphological Identification of *T. evansi*:

The detection then characterization of morphological characters of *Trypanozoon* in fresh wet and stained thick blood smears in camels (field isolates) was found slender parasites with an average of 21 µm in length. In addition, each had an undulating membrane, sub terminal kinetoplast and free flagellum (Photomicrograph 1). These morpho-metrics were considered characteristic of *T. evansi*.

Serological Identification of *Surra* in Camels with regards to Clinical Phase of Infection:

The developed reactions in both ELISA (cut off value= 0.270) and CATT (+1, +2 & +3) and positivity degree in relation to locality and seasonality are illustrated (Chart 2 & 3). The degree of positive results was used as indication of infectivity status of the examined individuals following molecular confirmation of infection and characterization of *T. evansi* isolates and quantification of cytokines expression

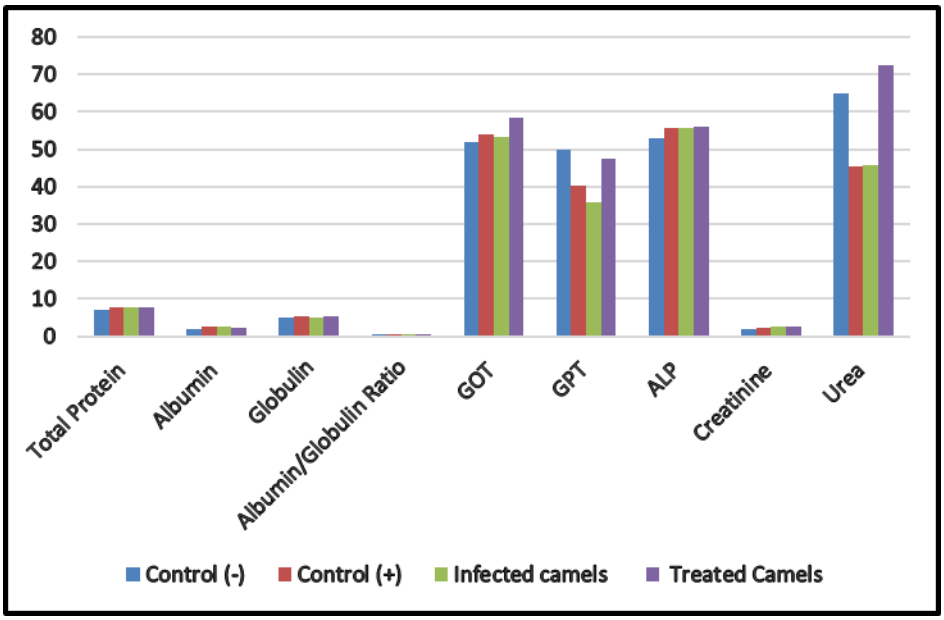
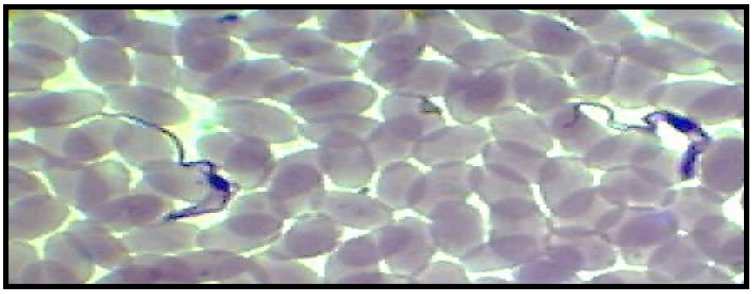


Chart (1): Blood biochemistry of *Surra* infected camels.

Table (4): Hematological parameters of *Surra* infected camels (Mean ± SD).

Parameters	Control (-)	Control (+)	Infected camels	Treated Camels
RBCs ($\times10^{6}$)	5.02±0.05	5.95±0.23**	5.05±0.3**	5.01±0.1**
Hb (g/dl)	13.92±0.58	19.13±1.05**	20.2±1.05**	14.01±0.54**
PCV (%)	40.75±1.75	55.00±2.89**	55.00±2.89**	39.05±1.75**
MCV (fl)	80.75±1.68	90.56±2.41*	92.4±2.35**	79.9±1.68**
MCH (pg)	27.72±0.49	31.62±0.75**	32.7±0.65**	27.02±0.54**
MCVC (g/dl)	34.46±0.40	33.78±1.01	34.98±0.98	33.09±0.39
Platelets ($\times10^{3}$)	95.16±1.98	96.66±2.59	97.16±1.99*	94.98±1.90*
WBCs ($\times10^{3}$)	11.6±0.29	9.15±0.51**	9.25±0.47**	11.1±0.31**
Neutrophils (%)	81.75±0.65*	83.20±0.59*	84.30±0.61*	80.55±0.65*
Lymphocytes (%)	11.77±0.43	11.40±0.56	11.55±0.51	11.07±0.39
Monocytes (%)	5.02±0.32	4.05±0.29	4.00±0.28	4.96±0.29
Eosinophils (%)	1.55±0.09	1.54±0.04	1.53±0.2*	1.55±0.01

* = significant at P< 0.05 ** = high significant at P< 0.01



Photomicrograph (1): *Trypanosoma evansi* between red blood cells in stained smear of camels by Field's Stain (x1000).

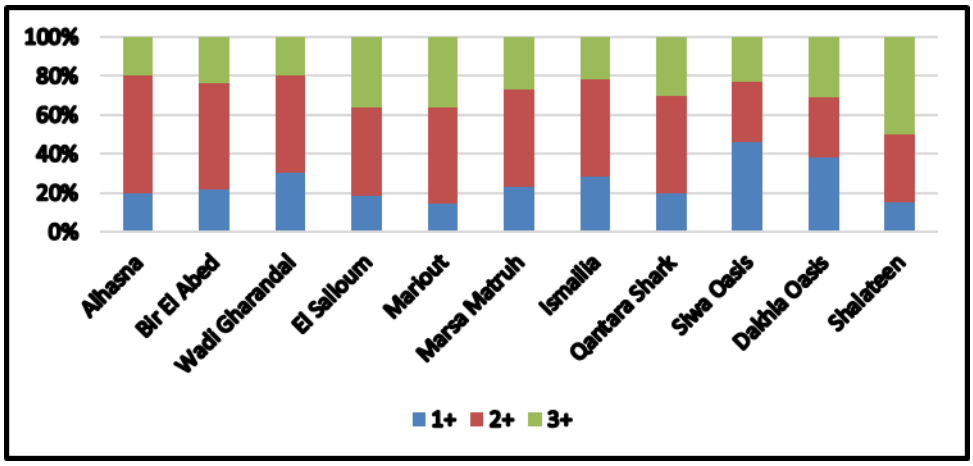


Chart (2): *Surra* serological diagnosis by CATT in Camels' regarding the incidence of each grade of positives results within each investigated locality.

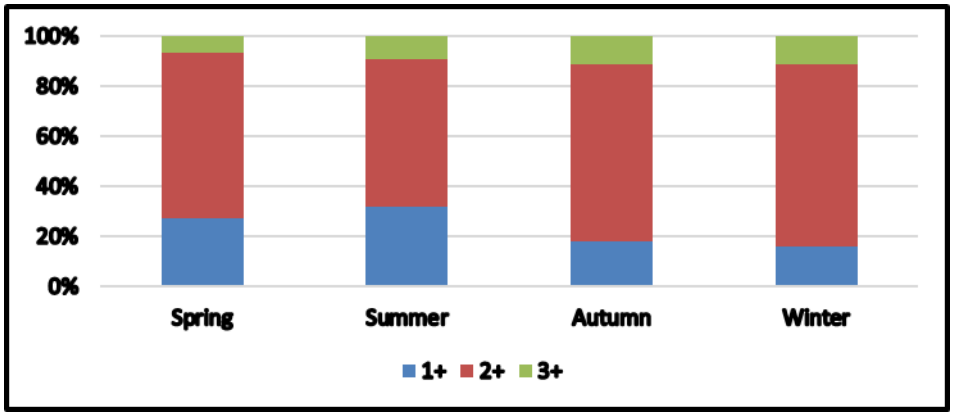


Chart (3): *Surra* serological diagnosis by CATT in Camels' regarding the seasonal incidence of each grade of positive results.

Identification of *T. evansi* the *Trypanozoon* species inducing Camels *Surra* by PCR:

Trypanozoon species molecular characterization in camels blood samples utilizing the specific primers of ITSI and SND, revealed the specified length of amplified fragments of both genetic regions; 580 bp and 164 bp, respectively (Fig 1: A & B), the identities of the sequences confirmed the morphological classification of *Trypanozoons* to their designed species; *T. evansi,* with similarities presents ranged from 97-100 % to records in the GenBank databases.

Efficiency of Diagnostic Techniques in *Surra* Incidence Determination with regards to Clinical Phase of Camels' Infection:

Trypanosoma evansi were detect in blood by wet blood smears 6.7% (5.36±1.185), field stained blood film 7.72% (6.18±1.387), and hematocrit centrifugation 14.66% (11.73±1.453) within specimens of camels from investigated localities; the gold standard parasitology diagnostic techniques (Table 5). The detection thresholds of these techniques were High parasitaemia dependent (≥ 40 trypanosome/field), thus were successful in acute infections only (Table 6). On the other hand, serological diagnosis of infection by ELISA; using homemade local *T. evansi* lysed isolate, as well as, commercially available CATT test revealed 44.1% (34.00±5.140) and 41.36% (35.27±4.760) seropositive incidence for *surra* (Table 5), respectively. CATT results represented 23.3%, 53.4% and 9.7% overall incidence for grades +1, +2 and +3, respectively. However, the incidence of each grade with regards to locality and seasonality is illustrated in charts (2 & 3).

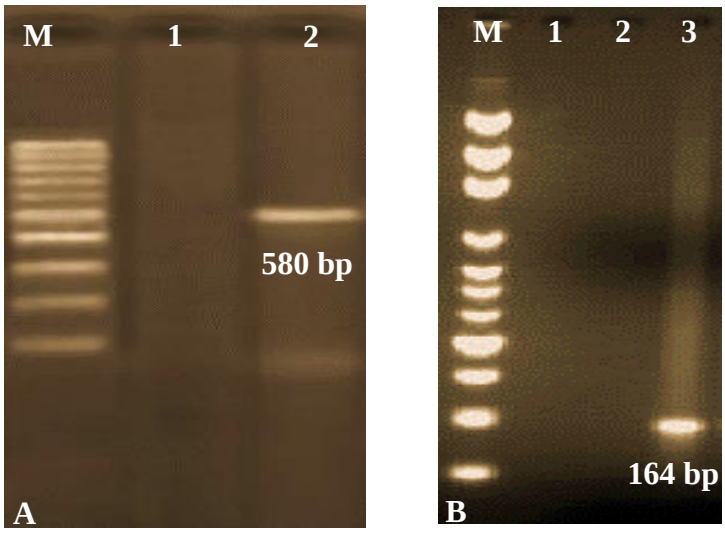


Figure (1). Molecular Detection and Characterization of *T. evansi* by PCR amplification of DNA markers ITSI and SND, the purified bands were visualized in 1.5% agarose gels stained with ethidium bromide.

A) Lane M: 100 bp DNA ladder, lane 1: Control negative, lane 2: 580 bp of ITSI fragment.
B) Lane M: 100 bp DNA ladder, lane 1 & 2: Control negative, lane 3: 164 bp of SND fragment.

Table (5): *Trypanosoma evansi* incidence within Infected Camels in the Egyptian desert with regards to the diagnostic test applied.

Method	DF	Mean	± SE		S.O.V
W.B.S.	5	5.36±1.185	1.185	**c**	**
F.S.S.		6.18±1.387	1.387	**c**	
H.C.T.		11.73±1.453	1.453	**c**	
ELISA		35.27±4.760	4.760	**b**	
CATT		34.00±5.140	5.140	**b**	
PCR		54.64±4.845	4.845	**a**	

Means with the same letter in the same column are not significantly different P≤0.01= ** highly significant between methods, DF= Degree of Freedom, SE= Standard of Error, S.O.V= Source of variance.

PCR screening of camels samples utilizing the specific primers of ITSI and SND, revealed 68.3% (54.64±4.845) overall *surra* incidence in examined blood. The significance of the PCR as diagnostic test in comparison to other diagnostic tests is illustrated in table (5). The incidence of camels *surra* calculated for locality and seasonality revealed from PCR obtained results were illustrated in tables (6, 7 & 8).

Justification of Diagnostic Data from Clinical Merits regarding Camels Groups:

The *trypanosomes* count/field during parasitological diagnosis had classified camels in to 4 groups: *surra*-free (control negative), acute infected (control positive group and infected group), sub-acute infected, and chronic infected (Table 10). The degree of developed reactions in ELISA and CATT diagnostic tests was used as indication of infectivity status of the examined individuals following molecular characterization of *T. evansi* isolates and quantification of cytokines expression (Table 9). Treatments were applied on all infected groups, and the obtained results were normalized by both camels' controls groups (Table 10).

Despite the recorded specificity of ELISA and CATT as screening tests, yet false positive results were recorded (Table 10). Hence, the positive reaction of grade (1) was noted in *T. evansi*- free, acute, sub-acute and chronic infected camels (Table 10).

Table (6): *Trypanosoma evansi* infection seasonal incidence within Camels in investigated localities of Egyptian desert by PCR.

Regions	Locality	Camel No. = Locality No. (11)× Individual No. (10)× Season (4)× year (2)									Seasonal Incidence of Infection	
		No.	A.	%	Sp.	%	Su.	%	W.	%	No.	%
Middle Sinai	Alhasna	80	5	0.83	18	3.00	30	4.99	7	1.16	60	9.98
Nourth Sinai	Bir El Abed	80	8	1.33	20	3.33	37	6.16	12	2.00	77	12.81
Southern Sinai	Wadi Gharandal	80	6	1.00	14	2.33	25	4.16	5	0.83	50	8.32
North West Coast	El Salloum	80	9	1.50	17	2.83	27	4.49	7	1.16	60	9.98
	Mariout	80	9	1.50	20	3.33	25	4.16	9	1.50	63	10.48
	Marsa Matruh	80	9	1.50	19	3.16	27	4.49	8	1.33	63	10.48
Ismailia	Ismailia	80	5	0.83	12	2.00	24	3.99	4	0.67	45	7.49
	Qantara Shark	80	4	0.67	13	2.16	25	4.16	3	0.50	45	7.49
Western Desert	Siwa Oasis	80	3	0.50	7	1.16	16	2.66	2	0.33	28	4.66
New Valley	Dakhla Oasis	80	2	0.33	9	1.50	20	3.33	2	0.33	33	5.49
South East Coast	Shalateen	80	8	1.33	20	3.33	37	6.16	12	2.00	77	12.81
Total		880	68	11.31	169	28.12	293	48.75	71	11.81	601	
D.F.	30											
Chi-Square (X)	13.08											
Prob.	Non-significant											

Table (7): *Trypanosoma evansi* infection seasonal incidence within Camels by PCR.

Season	DF	Mean± SE	S.O.V	
Autumn	3	6.182±0.772	**c**	**
Spring		15.364±1.403	**b**	
Summer		26.636±1.903	**a**	
Winter		6.455±1.090	**c**	

Means with the same letter in the same column are not significantly different, P≤0.01= ** highly significant between seasons, DF= Degree of Freedom, SE= Standard of Error, S.O.V= Source of variance.

Table (8): *Trypanosoma evansi* incidence within Camels in the Egyptian desert with regards to localities.

Regions	Locality	DF	Mean± SE	S.O.V	
Middle Sinai	**Alhasna**	10	15.000±5.759	bc	**
Nourth Sinai	**Bir El Abed**		19.250±6.421	a	
Southern Sinai	**Wadi Gharandal**		12.500±4.628	bc	
North West Coast	**El Salloum**		15.000±4.546	bc	
	Mariout		15.750±4.029	ab	
	Marsa Matruh		15.750±4.498	ab	
Ismailia	**Ismailia**		11.250±4.608	cd	
	Qantara Shark		11.250±5.105	cd	
Western Desert	**Siwa Oasis**		7.000±3.189	e	
New Valley	**Dakhla Oasis**		8.250±4.250	de	
South East Coast	**Shalateen**		19.250±6.421	a	

Means with the same letter in the same column are not significantly different, P≤0.01= ** highly significant between Localities, DF= Degree of Freedom, SE= Standard of Error, S.O.V= Source of variance.

Table (9): Relative up regulation in cytokines genes expression in acute *Surra* in Camels investigated during the present study.

Gene	DF	Means± SD2^{-}($\Delta\Delta$CT)	S.O.V	
IL-4	10	2.033 ± 0.020	**c**	**
IL-6		4.415 ± 5.178	**b**	
IL-8		11.69 ± 4.146	**a**	
IL-10		13.04 ± 7.103	**c**	

Table (10): Clinical phases of *Surra* in Camels regarding IL4, IL6, IL8 and IL10 quantitative expression as analytical biomarkers of infection.

Camels Group Designation *	*T. evansi* Diagnosis						Quantitative Cytokines Genes Expression (qRT-PCR)				Response to Treatment ¥	Clinical Phase of *Surra* in Camels
	Wet Blood Smears	Stained Blood Smears	Hematocrit Centrifugation	ELISA	CATT grade	PCR	IL4	IL6	IL8	IL10		
Control negative	-	-	-	+	+1	-	Normal				NA	Parasitaemia free, Aberrantly healthy
Control positive	+	+	+	+	+3	+	Up Regulation					High parasitaemia, Shedding parasite, Severe Symptoms
	10^7 trypanosomes										NA	
Acute Infection	+	+	+	+	+3/+2	+	Up Regulation					High parasitaemia, Shedding prarsite, Severe Symptoms
	≥10^7 trypanosomes										+3	
	+	+	+	+	+1	+						High parasitaemia, Shedding prarsite, Symptomless
	≥ 40 trypanosome/field										+3	
Sub-Acute Infection	-	-	+	+	+1	+	Weak Up Regulation				+2	Low parasitemea), Shedding prarsite, Symptomless, Aberrantly healthy
	≤ 40 trypanosome/field ᵅ											
Chronic Infection	-	-	-	-	+1	±	Down Regulation				+1	Parasitaemia free, Aberrantly healthy
	≤ 40 trypanosome/field ᵝ											

*****Camels were classified into groups according to their infection status as indicated by parasitological, serological, and molecular diagnostic tests.
ᵅ The hematocrit centrifugation was applied for concentration of *T. evansi* then parasitological diagnostic tests were applied on camels blood.
ᵝ The mice inoculation was applied for enrichment and concentration of *T. evansi* (Data not shown) then repeat the diagnostic techniques on mice blood.
¥ Treatemnt grade: NA= not applied, 3+= respond effectivelly, 2+= needed repeatedness of treatment, 1+= despite being Aberrantly healthy they did not respond to treatment hence resistance to drug had started.

Moreover, both tests failed to discriminate between active and previous infections phase in studied population of camels (Table 10). PCR is *T. evansi* –dependent, thus false positive were seldom, therefore, was considered the most specific and sensitive detection method (Tables 5, 6, 7, 8 and10).

Cytokines Expression in Camels regarding the Clinical phase of *Surra*:

The qRT-PCR results presented up regulation in cytokine genes expression during acute infections in individuals with high parasitemia; IL-4, IL-6, IL-8 and IL-10 (Means SD. $2^{-}(\Delta\Delta ct)$, but not for chronic infections (Table 9). Despite that sub-acute infected individuals showed fold increase in cytokines genes expression, but were lower than those recorded for acute infections. However, the fold increased were higher for IL-8 and IL-10 than those recorded for IL-4 and IL-6 (Table 9). The reaction efficiency for quantified genes of interest ranged between 0.95 and 1.03. The results were interpreted in association to diagnostic techniques applied, clinical phase of infections, and response to treatment-applied (Table 10).

DISCUSSION

Clinical infections of *T. evansi* are found under more than 30 vernacular names, however, disease severity is host-strain interaction dependent due to difference in host susceptibility and trypanosomes pathogenicity (Atarhouch et al., 2003; Antoine-Moussiaux et al., 2007; Amoudi et al., 2011; Haridy et al., 2011). There are no prospects for the development of new anti-trypanosome drugs (Abdille et al., 2008a & 2008b; Amer et al., 2011; Salim et al., 2011). Therefore, control of the disease remains a challenge, as chemotherapy has only modest success in treatment; which was noted in sub-acute group in the present study. Additional, the emerging trypanosomes resistance to current drugs was increasingly becoming a major problem which was noted in the chronic infected camels during the present study.

Epidemiologically, trypanosomes have the capacity for antigenic variation; hence, their ability to escape the triggered immune response compelling the host to mount a new cycle of antibody production each time a new variant appears (Donelson, 2003; Desquesnes et al., 2007; Yang et al., 2007). On the other hands, cross-immunity against any homologous and/or some heterologous stock infections, especially those which are drug resistant strains, were possible (Uzcanga et al., 2002; Camargo et al., 2004;Zayed et al., 2010a and 2010b; Milocco et al., 2013). Moreover, strains of *T. evansi* from all geographical regions and hosts present very similar zymogene (Amer et al., 2011; Salim et al., 2011). Consequently, the presented results were in agreement with Broadhead et al., (2006), Abdille et al., (2008a & 2008b), Laha and Sasmal, (2008), and Zayed et al., (2010a and 2010b) who stated that irrespective of host species, trypanosomes within the same geographical region have a similar morphometric parameters, genomics and proteomics profiles with a few very minor differences that were parasite-dependent affecting the hosts' developed immune reaction.

Hematological and biochemical profiles in studied camels revealed that macrocytic anemia and leucopenia were recorded in diseased camels but returned to normal parameters post treatment, while no significant differences were reported in biochemical changes between diseased and treated camels. As far as these results were released, there were no enough reports on hematological and biochemical parameters in camels' suffered or treated from *surra*. Therefore, experimental studies applied on camels with similar design to verify the obtained results are needed. The infection with *T. evansi* in camels was expected but the prevalence and distribution need to be investigated regarding the efficiency of diagnostic technique, in particular to evaluate exposure risk (Sarataphan et al., 2007; Desquesnes et al., 2009).

Still the parasitological diagnostic test is the golden techniques in *T. evansi* diagnosis, despite the biohazards of handling infected blood specimens. They were capable of classifying the clinical stage of infection according to *trypanosomes* numbers/blood field (Zayed et al., 2010a & 2010b; Milocco et al., 2013). According to manufacturer, CATT positive samples should mostly be observed during active infection; parasitaemia (Donelson, 2003; Desquesnes et al., 2007; Yang et al., 2007). Unexpectedly, some of the CATT positive samples were PCR negative in the present study. A proposal of recovered previous infections could explain these results, however, this probability is low (Zayed et al., 2010a & 2010b; Milocco et al., 2013). Consequently, this observation did not confirm the advantages of the CATT. Hence, the research team had expected CATT results to be simultaneous with ELISA; therefore, serological screening would help to select a sub-set of

samples for PCR which was not completely fulfilled. Never the less, PCR is still more efficient in diagnosis, but for practical and economic reasons, traditional PCRs could not be carried out during epidemiological surveys. Instead ELISA could partially fulfill the screening job despite the biohazard of handling local isolate to prepare the whole lysate antigen (Zayed et al., 2010a & 2010b; Milocco et al., 2013). In addition, its value should be further evaluated under experimental and natural infections before being accepted as efficient screening tool. The development of new molecular and serological tools would be very useful especially to generate data on potential of infections.

DNA technology had positively improved species identification of genus *Trypanosoma*, especially the pathogenic clades, yet more improvements are fundamental (Masiga et al., 1992; Bromidge et al., 1993; Desquesnes et al., 2001 & 2002; Milocco et al., 2013). Phylogenetic analysis of the structural genes in genus *Trypanosoma* identified three major clades including the "brucei-clade" to which *T. evansi* belongs, that includes all mammalian salivarian trypanosomes (Desquesnes et al., 2007; Laha and Sasmal, 2008). Identifying the molecular basis of such semilarity should be on the top of the proiority of the scientific comunity in the near future. Hence, the amplified sequences from investigated blood samples were belong to *T. evansi*, as well as other products with sequences specific to camels. Therefore, in the present study PCR results were sequenced to confirm the results obtained with TRYP1 primers; *Trypanozoon* sub-genus specific primers were applied and proved their specificity and sensitivity (Pruvot et al., 2010; Milocco et al., 2013). The satellite DNA fragments obtained by TBR show the classical pattern expected for *T. evansi* satellite DNA; confirmed by sequencing and nucleotide-BLAST comparison of amplified fragments. In our study, the 164 bp product obtained with TBR primers were interpreted as *T. evansi* DNA specific indicating *surra* in camels and confirming the diagnosis by parasitological and serological technology; a single-product with species-specific primer set detecting *T. evansi* in camels' samples. Hence, of the 601 samples testing positive for *T. evansi* with TBR primers, only 240 samples were positive with TRYP1 primers. These results are in agreement with previous reports because the target DNA is highly repetitive satellite DNA (10 000–20 000 copies), while ITS1 rDNA is repeated only 300–500 times; therefore, TBR test is more sensitive than TRYP1 test (Desquesnes and Dávila, 2002; Pruvot et al., 2010; Milocco et al., 2013). On the other hands, future novel molecular tools that could specifically detect and identify several *Trypanosoma* sp. in a single reaction will be appreciated.

The clinical signs of *surra* shown to be strong in camels, equids and carnivores (Sarataphan et al., 2007; Zayed et al., 2010a & 2010b). However, the medical and economic impacts should not be neglected in other host species. *Surra* prompts loss of production, even with sub- acute phase of the disease. Hence, the immunosuppressive effects of *surra* could prime either the development of inter-current diseases or vaccination failures (Onah et al., 1997; Holland et al., 2001; Singla et al., 2010; Zayed et al., 2010a & 2010b; Milocco et al., 2013). It is very well established that proper treatment/prophylactic programs is dependent on early efficient detection of infection, as much as, sensitive specific follow up of clinical cases with differential diagnostic capability; first onset and complete recovery from infection (Brown, *et al.,* 1990; El-Amin et al., 1998). Cytokines are one of the sensitive means to evaluate the functionality of the immune responses of animal host. Hence, they could serve as a suitable tool for health control and assessment of treatment and/or vaccine efficiency (Almeida et al., 2011; Hogeveen et al., 2011; Günther et al., 2016; Allam et al., 2017). Quantification of transcription levels of gene responsible for immunity well justify abnormal alterations in regulation due to infection (Fonseca et al., 2009). Mammalian cytokines, especially IL-4, IL-6, IL-8 and IL-10 had been considered useful markers in defining immune defenses triggered by *trypanozoons*-dependent expression (Bannerman et al., 2004). Blood included several leucocytes types; neutrophils, macrophages, lymphocytes, monocytes and natural killer (NK) cells (Bannerman et al., 2004). From the obtained qRT-PCR, *surra* could up regulated mRNA transcription of all investigated cytokines, especially IL-8 and IL-10 over than IL-4 and IL-6 in peripheral blood leukocytes. These cytokines induced a shift in T-cell phenotypes from CD4+ to CD8+ T-cells, as well as, differentiation of Th1 and Th2 lymphocytes to switch on the cellular immune cascades. In addition to, enhancing the humeral immune response (Fonseca et al., 2009). The IL-10 found capable to inhibit natural killer cells, so its higher expression usually indicates recurrent and/or chronic infectious pathogens (Riollet et al., 2001). Moreover, IL-4 is antagonist

to IFN-γ, therefore, it effectively regulated humeral IgE-mediated immune responses (Fonseca et al., 2009). Migration of neutrophils from blood stream to the site of infection is IL-8 dependent (Ribeiro et al., 1991; Galligan and Coomber., 2000). The presented results were in agreement with Galligan and Coomber, (2000), Lee et al., (*2003*), and Fonseca et al., (2009) who mentioned that expression of IL-4, IL-6 IL-8, IL-10 and interferon-γ (IFN-γ) was increased upon acute infections. The portrait were reversed during sub-acute and chronic infections. The presented study illustrated the capability of the investigated cytokines as biomarkers of clinical stages of *surra* in camels. However, experimental studies are fundamental to validate the obtained results and define the expression fold increase/decrease in relation to infection stages.

Finally, there is a wide range of genes which may play a role in the efficiency of immune reaction associated with *surra*, however, their inclusion was beyond the scope of the current study.

CONCLUSION

The results of this study indicate that camels' isolates of *T. evansi* belong to homologous phylogroup/serogroups/clade that contained a variety of virulence associated traits. Further large scaled studies and detailed analysis of the data are necessary to identify the association between virulence factors of *T. evansi* affecting camels' immunity. In addition, to investigate other hosts and reservoirs for *surra*. On the other hand, the zoonotic potential of *T. evansi* should be considered. Investigation in rodents needs to be completed to evaluate their role as reservoirs of potentially pathogenic *trypanosomes* in the vicinity of humans and livestock.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

NATA designed and performleed the epidemiological analysis, molecular characterization (PCR & qRT-PCR), serological diagnosis, and animals' treatments also wrote the manuscript. FME performed parasitological diagnosis. SMMA performed statistical and data analysis. All authors read and approved the final version.

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