Subclinical infection of paratuberculosis among camels in Egypt

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Abstract: Understanding clinical pathology during progressive stages of infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), specially the subclinical stage, and finding suitable methods for its diagnosis are key to the control of Johne's disease in camels. A total of 100, apparently healthy, one humped Arabian camel *(Camelus dromedaries)* from 3 separate herds were examined in this study. Five subclinical she camels 1-3 years old were detected using Ziehl-Neelsen stained rectal scrapings and confirmed by PCR. The most important haematological and biochemical changes in camels during the subclinical stage of infection with Johne's disease were recorded, in comparison with that obtained from the contact normal camels .

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1. Introduction

Archeologists think that domestication of the Arabian camels took place in the middle or southern part of the Arabian Peninsula about 3,000 B.C. From there, they moved to other parts of the Middle East and eventually into North Africa.

Several international and regional research projects targeted towards the study of camel diseases, and some stated that camel is less susceptible to diseases than other livestock **(Saint-Martin *et al.,* 1992**). In fact, the camel was found to be more susceptible than other animals to certain diseases, such as paratuberculosis (Johne's disease), clostridial enterotoxaemia and brucellosis (**Wernery and Kaaden, 2005**).

In this present work, we will focus on the subclinical form of **Johne's disease** infection which is a bacterial serious disease of domestic and wild ruminants causing severe economic losses, in camels that were infected years earlier **(Ayele *et.al.,* 2001 and Harris & Barletta, 2001).**

Mycobacterium avium subspecies Paratuberculosis (MAP), the causative agent of Johne's disease is a slowly growing acid fast microorganism that, unlike other cultivable mycobacteria, requires exogenous supplementation with ferric mycobactin for growth in primary culture **(Chiodini *et al.,* 1984 and Manning & Collins, 2001).**

The gastrointestinal tract is the primary site of infection (predominantly the small intestine). Young animals (up to few months of age) acquire the infection by ingestion of M. Paratuberculosis in feed or milk **(Ayele *et al.,* 2001)**. Adult animals that not manifesting clinical signs of the disease shed the causative agent in the milk **(Streeter *et al.,* 1995)**. The disease can also be passed on to fetuses by intrauterine **(Sweeney, 1996**). M. Paratuberculosis is excreted at high levels in the faeces which reflect high number of organisms in the intestinal wall.

As this disease has recently been diagnosed among dairy cattle herds in Egypt causing severe economic losses **(Salem *et al.,* 2005)** and may of human health significance **(Alluwaimi, 2007 and Behr & Kapur, 2008)** and due to shortage of the available literature and the lack of data about the clinical pathology of MAP infection in camels in Egypt, the aim of this work was planned to diagnose subclinical form of this infection through :

- Detection of the acid fast MAP in camel rectal scraping using ZN staining techniques in apparently healthy camel herds and. Confirmation of the positive cases using PCR technique.
- Recording the most important haematological and biochemical changes in camels during the subclinical stage of infection with Johne's disease in comparison with the contact normal camels .

2. Material and Methods

Animals,

A total of 100, apparently healthy, one humped Arabian camel *(Camelus dromedaries)* from 3 separate localities were examined in this study according to **(Manning and Collins, 2001)**. Fifty sporadic camels from Cairo and 50 camels in two separate herds naturally grazing in the area of Sinai Desert, Egypt (Table No.1). All the animals aged between 1 and 3 years. They were examined microscopically for acid fast bacilli (AFB) in rectal scrapings stained with Ziehl-Neelsen stain (ZN). Two categories were selected for sampling in this study; normal camels (n = 50) were clinically normal and negative for *M. avium* subsp. *Paratuberculosis* acid

fast bacilli using ZN stain and subclinical camels (n = 5) were positive for *M. avium* subsp. *Paratuberculosis* acid fast bacilli using ZN stain and confirmed by PCR .

Rectal scrapings,

Rectal scrapings were collected according to **Sellon (2008)** using a long-handled spoon shaped curette from the rectal wall. The scrapings were sent, in 2ml disposable plastic tubes, to the laboratory as soon as possible where it processed and examined.

Ziehl–Neelsen staining technique,

Ziehl–Neelsen staining technique was performed according to **Manning and Collins (2001)**.

DNA extraction for Feces samples

DNA was extracted from fecal samples after modification to the protocol accompanied with QIAamp® DNA stool mini kit (Qiagen) according to **Salem (2009)**.

IS*900* nested PCR assay

The primer set used in this PCR was the primer pair **TJ1** GCT GAT CGC CTT GCT CAT, **TJ2** CGG GAG TTT GGT AGC CAG TA in the first-round PCR and primer pair **TJ3** CAG CGG CTG CTT TAT ATT CC, **TJ4** GGC ACG GCT CTT GTT GTA GT in the nested PCR using the conditions described by **Bull *et al.*(2003)**.

Hematological examination

Blood samples were collected in 5ml disposable plastic tubes coated with anticoagulant (EDETA) from 50 normal camels (clinically normal and rectal scrapings free of AFB), and 5 subclinical camels (clinically normal and rectal scrapings positive AFB). The blood samples were sent to the laboratory as soon as possible and examined on the same day of collection. Haematological parameters were estimated with veterinary hematology analyzer (Vet Scan HM5) from ABAXIS Company, USA, according to **Pratt (1992)**. Mean corpuscular hemoglobin concentration (MCHC), were calculated from the erythrocytic series values according to **Dacie and Lewis (1991)**. Differential leucocytic count was carried out according to **Dessouky (2006).**

Blood samples were collected in 10 ml disposable plastic tubes without anticoagulant (EDETA) from 50 normal camels (clinically normal and rectal scrapings free of AFB), and 5 subclinical camels (clinically normal and rectal scrapings positive AFB). The blood samples were sent to the laboratory as soon as possible , serum was separated and stored in duplicates in 1ml disposable plastic tubes at - 20ºC until used for biochemical analysis **Pratt (1992)**.

Blood sera were used for determination of the serum concentration of blood urea nitrogen, creatinine, cholesterol, triglycerides, iron, copper, cobalt, sodium, inorganic phosphorus and Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ glutamyltransferase (γ-GT), Creatinine kinase (CK) activities, albumin and total protein. All biochemical analysis were carried out using specific kits produced by BioSystem Reagents and Instruments, Spain and the results were read at appropriate wavelength using the A 15 BioSystem Spectrophotometer (Automatic analyzer), TEUS00023 from BioSystem, Spain.

Chemical and solutions,

Chemical and solutions used for microscopical and hematological examinations as well as PCR technique were obtained from Merck Company – Germany**.**

Statistical analysis

The data were analyzed with Excel software and the results were expressed as mean ±SD. Differences between groups were analyzed using T test according to **(Perrie and Watson, 1999)**, $P < 0.01$ was considered statistically moderate significant and $P < 0.001$ was considered statistically high significant.

3. Results

Out of 100 examined apparently healthy camels in three different herds, 5 camels showed clumps of small strongly acid-fast bacilli in rectal scrapings stained with Ziehl-Neelsen stain (Table 1 and Figs. 1&2). All the 5 Ziehl-Neelsen positive camels when examined by PCR gave positive results, subclinical camels (Table 1).

Serum biochemical assays

Table (1): Subclinically positive camels in three different herds using rectal scraping stained with Ziehl-Neelsen stain and PCR.

Herd No.	Herd state	Locality	No. of animals in the herd	No. of +ve camels	
				Ziehl-Neelsen	PCR
1*	AH**	Giza	50	0	0
2	AH**	Sinai	24	2	2
3	AH**	Sinai	26	3	3
Total			100	5	5

* sporadic camels from Giza governorate, Egypt.

** Apparently healthy camels with no previous history of paratuberculosis infection.

A

B

Fig. (1): A- Positive camels in herd No. 2. B- Positive camels in herd No. 3

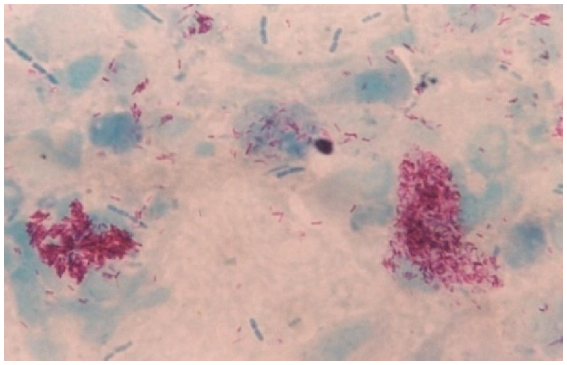

Fig. (2): Clumps of small acid-fast bacilli in Rectal scrapings from apparently healthy camels stained with Ziehl-Neelsen stain X1000

As shown in table (2); the mean of RBCs Count (10^6/µl), HB (g/dl), PCV (%), MCV (fl), MCH (pg), MCHC (g/dl) in normal 50 camels were 8 ± 1.9, 12.1 ± 1.2, 28 ± 1.1, 33.3 ± 2, 17.3 ± 3 and 48 ± 8.8 respectively. The mean of these parameters in the five subclinical camels were 5.6 ± 1.5, 9.1 ± 0.09, 23.2 ± 1.6, 29 ± 2, 16 ± 2 and 45 ± 5.1 respectively. Subclinical camels showed moderate significant decrease in RBCs Count, HB, PCV, MCV and MCHC values (P<0.01).

The mean of WBCs Count (10^3/µl), Lymphocytes (%), Neutrophiles (%), Monocytes (%), Esenophiles (%), Basophiles (%) and Platelets (x10^3) in normal 50 camels were 18 ± 2.1, 51 ± 5.1, 38 ± 7.3, 4 ± 0.5, 3 ± 1.2, 0.8 ± 0.2 and 290 ± 100 respectively. The mean of these parameters in the five subclinical camels were 19.8 ± 2, 50.3 ± 1.6, 36 ± 1.7, 15 ± 3.3, 2.8 ± 1.6, 0.6 ± 0.3 and 740 ± 120 respectively. Statistically, subclinical camels showed high significant increase in Monocytes (P<0.001) and moderate significant increase in WBCs Count and Platelets (P<0.01).

Table (2): Hematological Parameters of Normal and Subclinical Camels (Mean ± SD)

Parameter	Normal Camels(n=50)		Subclinical Camels(n=5)	
	Mean ± SD	Range	Mean ± SD	Range
RBCs Count (10^6/µl)	8 ± 1.9	7.5 - 9.8	5.6 ± 1.5*	3.7 – 6.3
HB (g/dl)	12.1 ± 1.2	11.1 - 14.8	9.1 ± 0.09*	8.1 - 9.8
PCV (%)	28 ± 1.1	26.2 - 29.1	23.2 ± 1.6*	21.2 - 24.5
MCV (fl)	33.3 ± 2	31 - 34	29 ± 2*	27.8 -30.1
MCH (pg)	17.3 ± 3	14.1- 19.2	16 ± 3	13.1 - 19.3
MCHC (g/dl)	48 ± 8.1	39.6 - 54.6	43 ± 4.1*	38 - 51.1
WBCs Count (10^3/µl)	18 ± 2.1	13.6 - 19	19.8 ± 2*	18 - 22
Lymphocytes (%)	51 ± 5	45.6 - 56.3	50.1 ± 1.3	45 - 55
Neutrophiles (%)	38 ± 7.3	30.1 - 45.8	36 ± 1.7	29 - 39
Monocytes (%)	4 ± 0.7	2,8 - 5.5	14 ± 3.1**	10 - 18.2
Esenophiles (%)	3 ± 1.2	1.0 - 4.3	2.8 ± 1.6	1.4 - 4.6
Basophiles (%)	0.8 ± 0.2	0.0 - 1.2	0.6 ± 0.3	0.0 - 1.1
Platelets (x10^3)	300 ± 60	230 - 380	750 ± 120*	600 - 890

Significance compared to the normal camel group *P<0.01, **P<0.001

As shown in table (3); the mean values of AST (IU/L), ALT (IU/L), GGT (IU/L), Urea (mmol/L) , Creatinine (mmol/L), Creatine kinase (CK) IU/L, Alkaline phosphatase (ALP) IU/L, Cholesterol (mmol/L) and Glucose (mmol/L) in the sera of 50 normal camels were 50 ± 22, 15.8 ± 3.7, 19.7 ± 3.2, 5.1 ± 0.6, 0.22 ± 0.03, 70.3 ± 15, 40.2 ± 10, 2.8 ± 0.3 and 6.2 ± 1.4 respectively. The mean of these parameters in the five subclinical camels were 70 ± 15, 18 ± 2.5, 21.3 ± 4, 5.3 ± 0.8, 0.18 ± 0.01, 72.1 ± 18, 42.2 ± 12, 2.0 ± 0.4 and 4.8 ± 1.1 respectively. Statistically; subclinical camels showed moderate significant increase in AST and moderate significant decrease in Glucose (P<0.01).

Table (3):Biochemical Parameters of Normal and Subclinical Camels (Mean ± SD)

Parameter	Normal Camels(n=50)		Subclinical Camels(n=5)	
	Mean ± SD	Range	Mean ± SD	Rang
AST (GOT) IU/L	50 ± 10	34.0 - 88.0	71 ± 22*	52 - 127
ALT (GPT) IU/L	15.2 ± 3.6	8.3 - 25.7	18 ± 1.1	15.7 - 30
GGT (IU/L)	19.5 ± 3.1	15.0 - 25.0	21.1 ± 4	18 - 25
Urea (BUN) mmol/L	5.3 ± 0.6	4.4 - 5.5	5.8 ± 0.8	4.5 – 5.3
Creatinine (mmol/L)	0.22 ± 0.03	0.12 - 0.35	0.18 ± 0.01	0.09 - 0.25
Creatine kinase (CK) IU/L	70.1 ± 13	31.0 - 86.0	71.1 ± 15	30 - 85
Alkaline phosphatase (ALP) IU/L	41.2 ± 13	30.0 - 53.0	41.2 ± 15	28 - 50
Cholesterol (mmol/L)	2.8 ± 0.1	1.5 - 3.3	2.0 ± 0.4	1.6 - 2.5
Glucose (mmol/L)	6.2 ± 1.4	3.0 - 8.9	4.1 ± 1.1*	2.5 - 5.1

Significance compared to the normal camel group *P<0.01

As shown in table (4); the mean values of total proteins (g/dl), albumin (g/dl), globulin (g/dl) and albumin / globulin ratio in the sera of 50 normal camels were 7.6 ± 0.9, 4.2 ± 0.5, 3.4 ± 0.2 and 1.2 ± 0.09 respectively. The mean of these parameters in the five subclinical camels were 7.3 ± 0.9, 3.5 ± 0.7, 3.8 ± 0.3 and 0.9 ± 0.07 respectively. Statistically; subclinical camels showed moderate significant decrease in Total proteins, moderate significant decrease in albumin and albumin / globulin ratio with moderate significant increase in Globulin (P<0.01).

Table (4): Serum Proteins in Normal and Subclinical Camels (Mean ± SD)

Parameter	Normal Camels(n=50)		Subclinical Camels (n=5)	
	Mean ± SD	Range	Mean ± SD	Range
Total proteins (g/dl)	61 ± 0.1	54 - 69	54 ± 0.2*	49 - 55
Albumin (g/dl)	33 ± 0.2	28 - 36	22 ± 0.1*	20 - 23
Globulin (g/dl)	28 ± 0.1	22 - 30	33 ± 0.1*	30 - 34
Al / Glo ratio	1.2 ± 0.01	1.0 - 1.3	0.7 ± 0.01*	0.6 – 0.8

Significance compared to the normal camel group *P<0.01

As shown in table (5); the mean values of Fe (mmol/L), Cu (µmol/L), Ca (mmol/L), Iph (mmol/L), Na (mmol/L) and K (mmol/L) in the sera of 50 normal camels were 22.5 ± 4, 0.94 ± 0.09, 2.1 ± 0.1, 1.8 ± 0.3, 163 ± 16 and 4.0 ± 0.6 respectively. The mean of these parameters in the five subclinical camels were 18.1 ± 3.6, 0.55 ± 0.08, 1.8 ± 0.2, 1.4 ± 0.3, 159 ± 19 and 3.8 ± 0.4 respectively. Statistically; positive contact camels group showed moderate significant decrease in Fe, Cu, Ca & Iph (P<0.01).

Table (5):Minerals in sera of Normal and Subclinical Camels (Mean ± SD)

Parameter	Normal Camels(n=50)		Subclinical Camels(n=5)	
	Mean ± SD	Range	Mean ± SD	Range
Fe (mmol/L)	23.2 ± 4	15 - 30	18.1 ± 3.6*	11.2 - 20
Cu (µmol/L)	0.96 ± 0.09	0.60 - 1.2	0.57 ± 0.03*	0.40 - 0.75
Ca (mmol/L)	2.1 ± 0.1	1.7 - 3	1.8 ± 0.3*	1.6 - 2.1
Iph (mmol/L)	1.8 ± 0.3	0.9 - 2.6	1.4 ± 0.3*	0.9 - 1.6
Na (mmol/L)	163 ± 16	100 -171	159 ± 19	100 - 166
K (mmol/L)	4.0 ± 0.6	2.5 - 5.2	3.8 ± 0.4	2.5 - 5.0

Significance compared to the normal camel group *P<0.01

4.Discussion

Mycobacterium avium subsp. *Paratuberculosis* (MAP) is the etiological agent of a severe gastroenteritis in ruminants, known as Johne's disease. It has significant impact on the global economy **(Salem *et al.*, 2005)**, and isolation of MAP

from intestinal tissue of Crohn's disease patients has led to concern that it may be pathogenic for humans **(Alluwaimi, 2007 and Behr & Kapur, 2008)**. Thus, the pathogenic role of *M. paratuberculosis* in camel population and its efficient control are topics of intense debate.

Whitlock and **Buergelt (1996)**, discussed the Subclinical stage of Johne's disease ; as a stage of animals age around 1- 2 years without any clinical symptom of paratuberculosis. At this stage of the infection animals shed the organism in undetectable level and could not be detected, cell mediated immune response (CMI) against *M. paratuberculosis* may be detected and only 15–25% of subclinical infected animals are detectable by faecal culture.

As shown in table (1), and figs. (1 & 2), Fifty sporadic camels, clinically normal and negative Ziehl-Neelsen stain for their rectal scrapings and negative PCR were considered as normal camels. Five asymptomatic camels, from the two separate distinct herds, positive Ziehl-Neelsen stain for their rectal scrapings and positive PCR were considered as subclinical camels.

Since treatment of paratuberculosis is expensive and unrewarding, diagnosis of the early infected and subclinical cases in the herds is an important goal of any effective disease control program for *M. paratuberculosis* **(Whittington and Sergeant, 2001)**. The effectiveness of diagnostic tools for the detection of animals infected with *M. paratuberculosis* is greatly affected by the biology of the disease. The progression from asymptomatic subclinical infection to a clinical disease state characterized by a classical protein-losing enteropathy corresponds with a concomitant shift from Th1-driven cellular immunity to Th2-mediated humoral immunity in the host.

Selected blood constituents were investigated in normal (n= 50) and subclinical (n=5) dromedary camels (*Camelus dromedaries*) infected with *M. avium* subsp. *Paratuberculosis*. The normal values for haematological estimations were in accordance with those reported by **Musa & Mukhtar, 1982; Baraka *et al.*, 2000; Feldman *et al.*, 2000; Peyssonnaux *et al.*, 2006 and AL-Busadah, 2007,** who recorded reference ranges for the haematological parameters of the one humped camel (Camelus dromedaries).

Observation of a deviation of certain haematological parameters from their normal limits often provides valuable information regarding health and sickness must be considered in diagnosis and treatment of camel diseases **(Kamal, 2008)**. The statistical analysis of the data presented in tables (2, 4 & 5) revealed that subclinical infected camels showed moderate significant decrease in RBCs count, HB, PCV, MCV and MCHC values (P<0.01); in comparison with the mean results obtained from normal camels. The obtained results indicated presence of a microcytic-normochromic anemia among the subclinical camels and a microcytic-hypochromic anemia among late subclinical cases, which help in the diagnosis of MAP infection during this asymptomatic stage of infection. These determined data agreed with **Weiss and Goodnough, 2005; Dessouky, 2006; Peyssonnaux *et al.*, 2006; Bhatt and Salgame, 2007; Roy *et al.*, 2007; Adamson, 2008; and Theurl *et al.*, 2008**, who explained that animals suffering from gastrointestinal infection, usually exhibit anemia and hypoglycemia. The principal feature of this anemia is reduction in the serum iron level (hypoferremia). It is known that, MAP infection is a gastrointestinal infection, invasion of MAP leads to activation of T cells (CD3+) and monocytes. These cells produce cytokines stimulate the hepatic expression of the acute-phase protein hepcidin, which inhibits duodenal absorption of iron. At the same time, they induce ferritin expression stimulates the storage and retention of iron within macrophages which lead to decrease iron concentration in the circulation and limite the availability of iron for erythroid cells **Beltan *et al.* (2000).** In addition, these cytokines directly inhibit the differentiation and proliferation of erythroid progenitor cells. As a result, there is iron-restricted erythropoiesis and, if severe, lead to production of microcytic, hypochromic red cells. This confirm the significant decrease in our obtained haematlogical and biochemical data.

Statistical high significant increase in Monocytes (P<0.001) and moderate significant increase in WBCs Count and Platelets (P<0.01) were detected among the subclinical camels. Moderate significant increase in WBCs Count, (P<0.01) and moderate significant decrease in Lymphocytes (P<0.01) were noticed in late subclinical camels (Table 2). These recorded results were combatable with those recorded by **Murray *et al.*, 1998 and Chaudhary & Iqbal, 2000**, who stated that, in *MAP* infected animals controlled with high levels of IFN-γ cytokine (subclinical cases), the leukocyte count remained steady at all times after infection, although the percentage of neutrophils increased during the transient phase of active infection. Inhibition of IFN-γ (late subclinical) is completely effaced by expansion of granulocytes with decrease in lymphocytes. IFN-γ stimulate platelet production by effects on megakaryocytes.

Biochemical analysis of blood is a valuable diagnostic tool differentiate between health and sickness of animals (Mohamed and Hussein, 1999). As regards to the statistical results of serum biochemical analysis shown in table (4); the recorded

mean values revealed moderate significant decrease in total proteins, albumin and albumin / globulin ratio as well as moderate significant increase in globulin (P<0.01) of the subclinical infected camels in comparison with the mean values of normal. The presented results are agree with that previously recorded by **Faye and Bengoumi, 1994; Mohamed and Hussein, 1999 and Kamal, 2008**, who demonstrated that, mean serum albumin concentration and A/G ratio in normal camels are significantly higher than those in other ruminants being more than one. This makes it possible to maintain the high colloid osmotic pressure needed for storing water in blood or regulating water balance. In gastrointestinal infection there are hypoproteinemia and changes in serum total protein, attributed to decrease of albumin and increase of globulins. The marked decrease of albumin due to leakage of albumin through damaged tissues and the increase of gamma globulins is a compensatory reaction to restore the reduced plasma osmotic pressure resulted from loss albumin. This can explain the obtained significant decrease in serum albumin of subclinically infected camels with *MAP*, and refer that serum protein analysis can be used as a diagnostic tool for detection of subclinical camels infected with *MAP*.

Regarding to the obtained mean values of serum biochemical analysis illustrated in table (3); statistical moderate significant increase in AST and moderate significant decrease in Glucose (P<0.01) were noticed in the sera of the subclinical infected camels with *MAP* than normal. The obtained results indicated liver affection and explained the decrease in serum proteins in subclinical infected camels. The presented results came in agree with that previously reported by **Sarwar *et al*., 1992; Bengoumi *et al.*, 1997; Mohamed and Hussein, 1999; and Kamal, 2008,** who stated that in gastrointestinal inflammatory disorders due to infection, regarding to the liver function parameters, an elevation in AST and ALT usually seen.

Essential trace elements are integral components of certain enzymes and important biological compounds that have major physiological roles, as iron in haemoglobin, cobalt in vitamin B_{12}. Regarding to the obtained statistical results illustrated in table (5); the mean values of Fe, Cu, Ca & Iph revealed moderate significant decrease (P<0.01) in subclinically infected camels. The obtained results regarding to the decrease in these essential trace mineral supported the obtained haematological changes and explain the cause of anemia in the subclinically infected camels. The presented results agree with that recorded by **Baraka *et al.*, 2000; Liu, 2003; AL-Busadah, 2007 and Kamal, 2008**, who stated that; *MAP* reactors always reveal decrease in the ferrous, copper and calcium values. Decrease in serum calcium levels concerned with hypoprotenemia as the serum calcium existed in protein-bound form that could not be repleanished by reabsorption.

5. Conclusions

Understanding pathogenesis during the subclinical stage of infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and finding suitable methods for its diagnosis are key to the control of Johne's disease in camels. In the present study, our investigations proved that determination of certain blood parameters as RBCs, Hb, PCV %, MCV, MCHC, WBCs Count, Monocyte % and Platelets; as well as, serum biochemical analysis for total proteins, Albumin, Globulin , Albumin / Globulin ratio, AST, Glucose, Fe , Cu , Ca and Iph; supported and agreed the obtained results of the ZN stained rectal scrapings and PCR reaction in the subclinically infected camel and can be used as a diagnostic tools for detection of the infected camels with *MAP* during its asymptomatic stage.

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