Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.071

RESEARCH ARTICLE

Molecular Characterization of *Cryptosporidium* Infection and Analysis of Hematological and Biochemical Changes in Diarrheic Pre-Weaned Calves in Egypt

Ayman Ahmed Shehata[1], Mahran Mohamed Abd El-Emam[2*], Heba Gouda[3], Basma Mustafa El-Said[3], Marwa B. Salman[4] and Eman Beshry Abd-Elfatah[1]

[1]Department of Animal Medicine, Infectious Diseases, Faculty of Veterinary Medicine, Zagazig University, El-Shohada, Moawwad, Qesm Awel AZ, Zagazig, 44511, Egypt; [2]Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Zagazig University, El-Shohada, Moawwad, Qesm Awel AZ, Zagazig, 44511, Egypt; [3]Department of Animal Medicine, Internal Medicine, Faculty of Veterinary Medicine, Zagazig University, El-Shohada, Moawwad, Qesm Awel AZ, Zagazig, 44511, Egypt; [4]Department of zoonotic diseases. National Research Centre. Post Box 12622, El Bohouth str.Dokki.Cairo, Egypt
*Corresponding author: mahranmohamed1234@gmail.com

ARTICLE HISTORY (23-186)

Received:	May 13, 2023
Revised:	July 17, 2023
Accepted:	July 31, 2023
Published online:	August 16, 2023

Key words:
Calves
Cryptosporidiosis
Hemato-biochemical Changes
Genotyping
Molecular Epidemiology

A B S T R A C T

Cryptosporidiosis is a major diarrheal disease with high mortality, primarily in young animals, and a zoonotic risk of infection. The molecular epidemiology patterns of *Cryptosporidium* isolates are still poorly understood in Egypt and other developing countries. The goal of this research was to better understand the genetic diversity of *Cryptosporidium* isolates in pre-weaned cattle calves in Egypt's Al-Sharkia governorate by examining the hemo-biochemical changes in infected calves. We used 100 diarrheal faecal samples to detect the presence of *Cryptosporidium* oocysts by Ziehl-Neelsen (ZN) staining. Twenty-two of the positive samples after staining were further verified using PCR targeting the *Cryptosporidium* oocyst wall protein (COWP) gene. We found that *Cryptosporidium. Parvum (C. parvum)* was the sole species identified using the polymorphic 60 kDa glycoprotein gene (GP60) locus. Sequencing GP60 locus samples revealed dominant circulation for subtype IIa of C. parvum in calves, unlike most previously reported sequences in Egypt. These sequences were 100% like those in Latin America (Brazil, KT948746 EPC30). Egypt imports cattle calves from Latin America, mainly Brazil, to overcome the deficiency in meat production, so it is more likely that the *C. parvum* sequences circulating in Egypt appear to be from different origins. Red Blood Cells (RBCs), Hemoglobin (Hb), Mean Corpuscular Hb (MCH), lymphocytes, and Total Iron Binding Capacity (TIBC) values were all significantly diminished in the infected calves than in the healthy ones, while Packed Cell Volume (PCV) and Mean Corpuscular Volume (MCV), neutrophil, eosinophil and monocytic percentages, Aspartate (Asp), and Glutamate Decarboxylase (GAD) values increased. Our study for the first time identified the presence of *C. parvum* isolates and changes in hemato-biochemical conditions in pre-weaned cattle calves in Al-Sharkia governorate, which may address the potential role of imported livestock in disease transmission.

To Cite This Article: Shehata AA, El-Emam MMA, Gouda H, El-Said BM, Salman MB, Abd-Elfatah EB, 2024. Molecular characterization of *Cryptosporidium parvum* infections and analysis of hemological and biochemical changes in diarrheic pre-weaned calves in Egypt. Pak Vet J, 44(1): 135-140. http://dx.doi.org/10.29261/pakvetj/2023.071

INTRODUCTION

Cryptosporidium species are primarily implicated as the most prevalent pathogen in calves compared to other enteric infections among the different invasive entero-pathogens (rotavirus, bovine coronavirus, and entero-toxigenic *Escherichia coli*) that produce diarrhea in calves (Karanis *et al*., 2010). The species in bovine hosts are characterised by specific age-related distribution with *Cryptosporidium parvum* (*C. parvum*) the most prevalent species in pre-weaned calves (1-3 months) in many countries and *C. bovis* and *C. ryanae* predominating in calves aged 3 - 11 months (Qi *et al*., 2020). Also, the infection caused by *C. parvum* is regarded as a high-risk zoonotic disease since the parasite mostly causes watery diarrhea in newborn calves and is easily transferred to

humans through contaminated consumables (Gamsjäger *et al*., 2023).

Routine microscopic diagnostic methods lack discriminatory ability between *Cryptosporidium* spp. due to similar morphologic features of *Cryptosporidium* oocytes. Therefore, the use of molecular techniques has received a lot of attention along with gene sequencing to distinguish between different species and subtypes of *Cryptosporidium* especially in a low fecal samples' density (Felefel *et al*., 2023). Generally, most of the recent research have either used PCR followed by sequencing or a combination of PCR and RFLP to target housekeeping gene loci such as the *Cryptosporidium* oocyst wall protein (COWP) gene. Analyzing the polymorphisms in the glycoprotein gene (GP60) was reported for subtyping research on *C. parvum* and *C. hominis* (Chen *et al*., 2023). Such molecular epidemiological studies are of high value for monitoring and tracking infection and spread of the parasite (Thompson and Ash, 2016).

Biometric indices are crucial to promptly assess the status of health for successful treatment and making a prognosis of diarrheic calves, in addition to epidemiologic investigations of diarrhea outbreaks to discover infections, their correlation with diarrhea, therapy, and control techniques (Muktar *et al*., 2015). Thus, hematological, and biochemical analyses are highly important tools in identifying diseases, medical decision making and providing an insight during the diagnostic procedure by comparing the values reported from diseased animal with normal values of healthy animals.

Reference intervals of complete blood cell count (CBC) for each animal species must be precisely set to evaluate the metabolic and health status of the animal (Song *et al*., 2020). Although the reference values for various blood variables, including serum biochemistry and bovine blood indices, are well-established for adult cattle, there is a clear gap in the data available for calves (Constable, 2004). Most of the published data concern the addition of amino acids to feed for healthy calves, but a few studies have observed anomalies in the plasma amino acids of diarrheic calves (Gerrits *et al*., 1998; Wang *et al*., 2012).

Although the prevalence and molecular distribution of *Cryptosporidium* species have recently been found to be increasing in Egypt's pre-weaned calves age group (Elmahallawy *et al*., 2022), very little is yet known regarding the genetic diversity of these animals as many earlier studies in Egyptian animals were primarily conducted microscopically (Naguib *et al*., 2018). Also, the adult cattle reference intervals have their limits and can be deceptive when used to assess young calves (Panousis *et al*., 2018). Only a few studies have looked at the blood parameters of native Egyptian calves in Egypt, and most veterinarians in the country manage cases of calf diarrhea by using clinical examination. Moreover, Al-Sharkia is one of the known Egyptian governorates for intensive animal farming.

This study had to primary objectives. The first was to molecularly characterise the circulating species and subtypes of *Cryptosporidium* in pre-weaned bovine calves in the region of Al-Sharkia utilising PCR amplification of the COWP gene and sequence analysis of the polymorphic *C. parvum* GP60 gene. The second was to measure selected haematological indices in diarrheic calves.

MATERIALS AND METHODS

Animals: A total of 100 diarrheic cattle calves showing signs suspected to be cryptosporidiosis in a private farm with a total of 183 pre-waned calves located at Al-Sharkia governorate, Egypt were examined during October 2022. Age of all diarrheic calves ranged from 3 to 30 d. General clinical examination was carried out according to Constable *et al*. (2017), to score body temperature, varying degrees of dehydration and weakness, and consistency of feces.

Samples collection: Fresh feces were taken directly from the rectum of each diarrheic calves and examined for cryptosporidiosis using Ziehl-Neelsen (ZN) staining. Blood samples were aseptically taken from *Cryptosporidium*-positive calves (n=22) and negative control groups (n=15). Blood was taken from each calf during the clinical evaluation and therapeutic process in October 2022, for the hematological and biochemical assays.

Laboratory methods

Ziehl-Neelsen technique (ZN staining): Detection of *Cryptosporidium* oocysts was done in all 100 faecal samples according to the method described by Fayer and Xiao (2007). Smears of concentrated fecal samples were stained by ZN method.

Molecular confirmation of cryptosporidiosis: Direct DNA extraction from 220mg of faecal samples was carried out using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Emerald Amp Max PCR Master Mix (Takara, Japan) was utilized for the PCR. A 553-bp fragment of the COWP gene was amplified using primers 5-GGACTGAAATACAGGCATTATCTTG-3 and 5-GTAGATAATGGAAGAGATTGTG-3 (Feltus *et al*., 2006). A total of 25µl reaction mixture was prepared, containing 12.5µl of PCR Master Mix (EmeraldAmp Max, Takara, Japan), 1µl of each primer of 20pmol concentration, 5.5µl of water, and 5µl of DNA template. PCR amplification was performed in a T3 Biometra thermal cycler using reaction conditions as described by Feltus *et al*. (2006).

Genotyping and subtyping of C. parvum isolates: All positive samples for *Cryptosporidium* COWP gene were further analyzed by nested PCR targeting *C. parvum* specific GP60 gene. Nested PCR was done as previously described with the primers 5-ATAGTCTCCGCTGT ATTC-3 and 5-GCAGAGGAACCAGCATC-3 used in the primary PCR to amplify approximately a 914 bp fragment and the primers 5-TCCGCTGTATTCTCAGCC-3 and 5-GAGATATATCTTGGTGCG-3 in secondary PCR a 850 bp fragment (Helmy *et al*., 2013).

Phylogenetic analysis: DNA cycle sequencing was carried out on an ABI 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan) with the ABI BigDye Terminator Mix ver. 3.1 kit (Perkin-Elmer). A BLAST® analysis (Basic Local Alignment Search Tool) was initially performed to determine sequence identity to GenBank

accessions. The final *C. parvum* sequences were given names based on the primary subtype family discovered by BLAST search and the nomenclature approach suggested by Sulaiman *et al*. (2005). A phylogenetic tree was created using the MegAlign module of Lasergene DNA Star version 12.1 Clustal *et al*. (1994), and the phylogenetic analysis was performed using MEGA6 according to Tamura (1992). The GP60 gene nucleotide sequences were deposited in GenBank under accession numbers OP750034, OP750035 and OP750036.

Hematological analysis: Hematological parameters such as Total Red Blood Cells (RBCs) Count, Total Leucocyte Count (TLC), Differential Leukocyte Count, Hemoglobin (Hb), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC) were measured using an auto-analyzer machine (Medinic CA 620 vet).

Biochemical analysis: A plasma sample was used to determine Total Amino Acid (TAA) concentrations using Total Amino Acids (T-AA) Colorimetric Assay Kit, provided by elabscience (Catalog No: E-BC-K055-M). Serum samples were collected to measure glutamic acid by glutamic acid ELISA Kit, provided by elabscience (Catalog No: E-BC-K118-S), aspartic acid according to the protocol of colorimetric/fluorometric assay kit (ApexBT). Total iron binding capacity (TIBC), Transferrin Saturation-Total Iron Binding Capacity (TS-TIBC), and serum iron were measured by BioVision's assay kit (Milpitas Boulevard, Milpitas, CA 95035, USA).

Statistical analysis: The statistical significance of the comparison between the two groups was assessed using Student's t-test. Statistical analysis was performed using Windows SPSS 16.0 (SPSS Inc., Chicago, IL). Results were presented as mean standard error of the mean (SEM), and statistical significance was determined by $P \leq 0.05$.

RESULTS

Clinical findings: General clinical examination revealed a morbidity rate of 54.6% (100/183) without any mortality case of pre-waned calves in the private farm at Al-Sharkia governorate, Egypt. Animals showed watery whitish or yellowish diarrhea and faeces contained blood or mucous in some calves. Semi liquid faeces (score 3) were recorded in 42 calves, while liquid diarrhea (score 4) was found in 58 calves. Also, varying degrees of decline in suckle reflex and dehydration, dullness, tenesmus and abdominal pain, pale mucous membrane were present. In most cases, the diarrhea abated 3-4 d following the symptomatic treatment though marked weight loss and dehydration were observed in cases with prolonged diarrhea for more than 7 d. All *Cryptosporidium* positive calves in this study were suffering from watery diarrhea.

Results of Zeihl Neelson (ZN) technique: *Cryptosporidium* oocysts were found in 22% (22/100) of the collected fecal samples. In ZN staining, the *Cryptosporidium* oocysts appeared red spherical or slightly ovoid and the sporozoites were arranged near the periphery within the oocyst and stained slightly darker. *Cryptosporidium* infection rate among age groups was statistically not significant (p= 0.562). Calves aged 3–7-day-old had 25% (10/40) of *Cryptosporidium* infections, 8–15-day-old calves 21.2% (7/33) and 16–30-day-old calves, 18.5% (5/27).

Molecular confirmation of *Cryptosporidium* infection: *Cryptosporidium* infection was confirmed in all ZN positive samples by PCR amplification of the ~553 bp fragment of the universal COWP gene.

Genotyping of *Cryptosporidium parvum:* All *Cryptosporidium* COWP PCR positive pre-weaned calves generated the expected ~914 bp fragment of *C. parvum* GP60.

Phylogenetic analysis for subtyping for *C. parvum* isolates: The nucleotide sequences data of the three *C. parvum* isolates were 100% like each other. Unlike most of GP60 *C. parvum* sequences reported in Egypt where IId family is highly prevalent in cattle calves, the three obtained nucleotide sequences corresponded to the most widespread subtype IIa family of *C. parvum* (IIaA15G1R1subtype) worldwide. Moreover, sequences of this study were found 100% identical to *C. parvum* isolates from different localities despite different host species and animal ages, such as isolates from neighboring countries (Israel, MW411017C4), and with that of Latin America (Brazil, KT948746 EPC30) isolated from one-year aged foals.

Changes in hematological parameters in infected calves: The hematological results of *Cryptosporidium* diseased and control calve groups are shown in Fig. 2. The data revealed a significant decline in the value of a total RBCs count in the diseased calve group than the control calve group (P=0.007) and marked decrease in the average Hb level in diseased calves group matched to control calves' group (P=0.0001). The mean percentage PCV value and MCV values showed significant increase in diseased calves' group than the average mean of control calves' group (P=0.001 and P=0.0009, respectively), while the mean of MCH was significantly decrease than control calves' group (P=0.0002). Additionally, a significant increase in the TLC of cryptosporidium calves' group compared to control calves' group (P=0.0001). The mean percentage neutrophils, eosinophils and monocytes values showed significant increase in diseased calves' group than control calves' group (P=0.0001, P=0.04 and P=0.01, respectively), while the mean of lymphocytes was significantly decrease than control calves' group (P=0.0006).

Changes in biochemical parameters in infected calves: TIBC values were considerably lower in infected calves compared to healthy calves (P=0.001), but plasma Asp and GAD were significantly higher (P=0.001 and P=0.0009, respectively) (Fig. 3). In terms of TAA, iron, or TS-TIBC, there was no statistically significant difference between the two groups (P=0.21, P=0.2 and P=0.06, respectively).

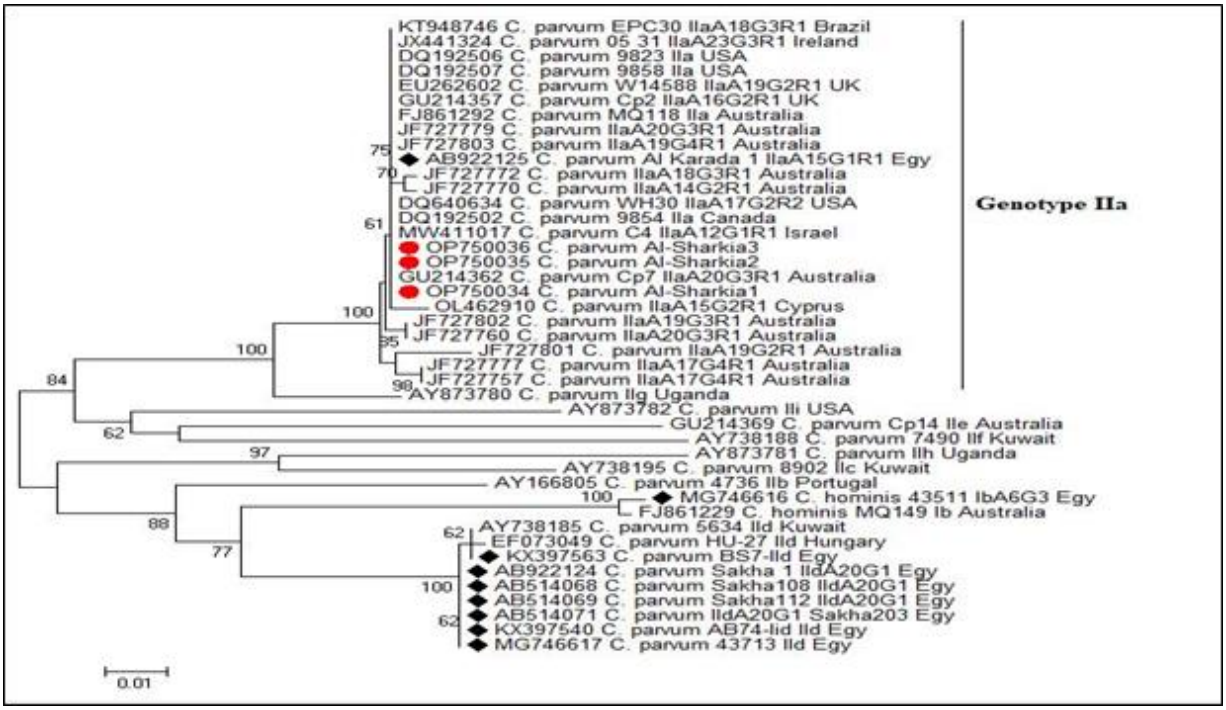

Fig. 1: The phylogenetic tree based on gp60 sequences for Cryptosporidium parvum. Red filled circles represent sequences of this study belonged to IIa subtype family, while Black rectangles are previous Egyptian gp60 sequences clustered mostly within subtype IId family.

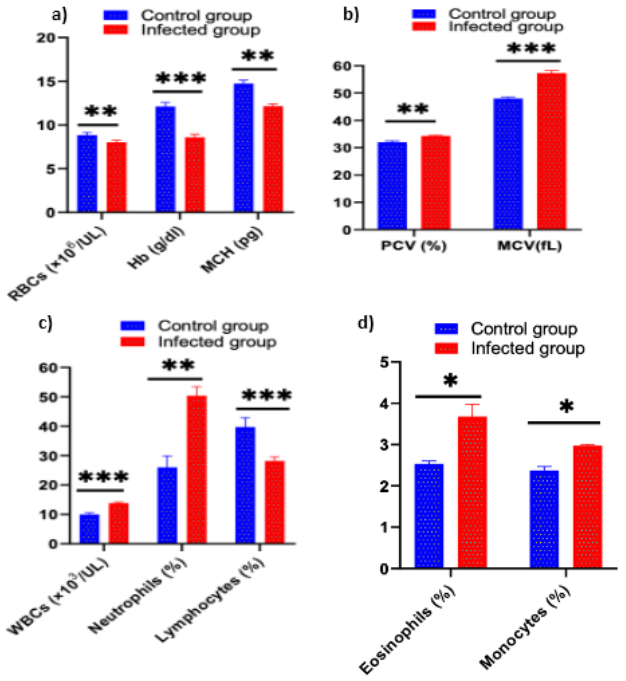

Fig. 2: Hematological parameters of diseased calves compared to the control healthy group: Data are presented as mean ± SD. *P < 0.05 vs control group. **P < 0.01 vs control group .*** P < 0.001 vs control group. *Hb: Hemoglobin; RBCs: Red Blood Cells; WBCs: Total leucocyte count; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration

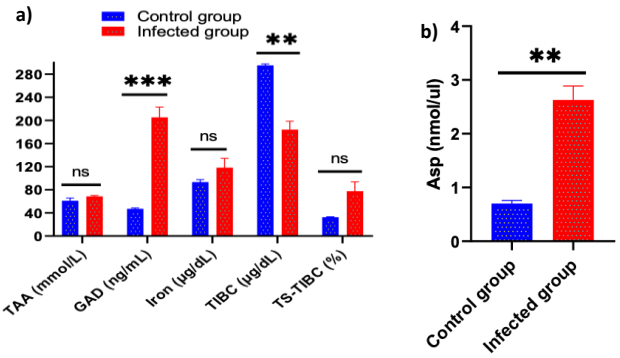

Fig. 3: Some biochemical parameters of calves suffering from cryptosporidiosis. Data are presented as means ± SD. *P < 0.05 vs control group. **P < 0.01 vs control group. *TAA: Total Amino Acid; Asp: Aspartate; GAD: Glutamate Decarboxylase; TIBC; Total Iron Binding Capacity: TS-TIBC; Transferrin- Total Iron Binding Capacity.

DISCUSSION

This study aimed to molecularly characterize *Cryptosporidium* species present in pre-weaned cattle calves at Al-Sharkia governorate, Egypt and determine hematological and biochemical parameters in diarrheic calves. *Cryptosporidium* oocysts were detected in 22 samples by microscopic ZN stain, and PCR targeting *Cryptosporidium* COWP gene confirmed the microscopic results. This *Cryptosporidium* infection rate of 22% was compatible with previous studies conducted in Egypt by Amer *et al*. (2010) who reported *Cryptosporidium* infection rate of 30% in cattle calves aged one month or less. Additionally, Taha *et al*. (2022) found a 49 % infection rate and discovered 100% identical C. parvum isolates from cows with two other isolates from water and humans, which is likely due to zoonotic transmission of Cryptosporidium in the research area. While other studies showed much lower rates of 6.90, 9.7 and 11.11% in cattle calves, respectively, by Mahfouz *et al*. (2014), Naguib *et al*. (2018) and Essa *et al*. (2014). Many factors can result in different *Cryptosporidium* prevalence rates within or between countries from population susceptibility due to age and immune status, housing density to management system of the target population.

All *Cryptosporidium* COWP PCR positive pre-weaned calves generated the expected ~914 bp fragment of *C. parvum* GP60 gene. These findings support the widely documented age-related distribution pattern that *C. parvum* is only present in calves younger than three months (Abdou *et al*., 2022). This study only targeted diarrheic cattle calves where only *C. parvum* causes the clinical illness in neonatal calves.

C. parvum isolates of this study were in the IIa family (IIaA15G1R1 subtype). However, this finding does not correspond previous findings in Egypt, where the IId subtypes are most common among cattle, with the IIdA20G1 subtype being endemic (Ibrahim *et al*., 2016).

Similarly, in human infections, subtype family IIa were frequently reported in Europe and USA (Iqbal *et al*., 2012) and in Kuwait (Majeed *et al*., 2022). Chen *et al*. (2023) indicated that IIa subtype is globally prevalent, while IId is found in Asia, Europe, and Africa and IIl is only found in Europe. Sequences of this study were found to be 100% identical to *C. parvum* isolates circulating in different industrialized localities despite different host species and animal ages, for example isolates from the neighboring country (Israel, MW411017C4), sequences of Latin America (Brazil, KT948746 EPC30), and that from UK, USA and Canada, GU214357 Cp2, DQ192507 9858, and DQ192502 9854, respectively (Ghaffari *et al*., 2014).

In the present investigation, the infected calves with cryptosporidiosis had significantly lower values of total RBCs count, Hb, and MCH and a markedly higher level of PCV and MCV than the non-infected calves. This result is in line with observations made by Mullakaev *et al*. (2020), who found that diarrheic buffalo calves had significantly lower Hb and RBC counts than healthy calves. Furthermore, Saleh *et al*. (2022) showed PCV values in diarrheal calves were higher than those in control, healthy animals. These results may be explained by hemoconcentration of blood caused by high body fluid loss and insufficient milk and fluid intake during diarrhea.

According to our data, *Cryptosporidium*-infected calves had higher TLC, higher neutrophilic, eosinophilic, and monocytic percentages, and lower lymphocytic values than non-infected calves. Similar findings were obtained by Brar *et al*. (2015), who found marked leukocytosis, neutrophilia, and lymphopenia in diarrheal calves compared to control ones. Gamsjäger *et al*. (2023) concluded that *C. parvum* infection provoking neutrophilic enterocolitis, may be due to the lack of fully innate gut defenses in neonatal calves. Likewise, eosinophilia was seen in cryptosporidiosis-infected calves as contrasted to healthy calves (Sahu and Maiti, 2011). This alteration may have happened because of normal body defense mechanisms against infection, dehydration, hemoconcentration and *Cryptosporidium* induced neonatal enteritis in calves.

Our results revealed that TAA values did not change in the two experimental groups. This finding was consistent with Tsukano and Suzuki's study, which found no statistically significant differences in plasma TAA concentrations between healthy and diarrheal calves (Tsukano and Suzuki, 2019). These alterations may be explained by the intestinal damage caused by *Cryptosporidium* infection in calves being not severe enough to affect the total amount of amino acids. Also, we found an increase in plasma aspartate level in infected groups compared to controls. The increase in concentration of plasma aspartate in our study has been mentioned in previous studies (Tsukano and Suzuki, (2019) and may be due to diarrhea accelerating the breakdown of histidine, glutamine, and arginine in the intestines, with the resulting glutamic acid transformed by AST and ALT into alpha-ketoglutarate, alanine, or aspartic acid. Moreover, our findings indicated that diseased calves had greater plasma levels of GAD than non-infected ones, which may be mediated by the significant loss of nutrients in the intestinal tract caused by diarrhea which result in drop of glutamic acid.

Our study revealed a non-significant increase in serum iron level and TS-TIBC and a significant decrease in TIBC in infected calves in relation to controls. Al-Laham *et al*. (2015) showed a reduction in TIBC in children suffering from diarrhea in Gaza. These results may have occurred due to the loss of total serum proteins and albumin in diarrheic calves and mucosal inflammation induced by *Cryptosporidium* infection. The non-significant increase in TS-TIBC in this study was due to a non-significant increase in serum iron level and a significant decrease in TIBC as TS% was calculated by dividing serum iron by serum TIBC.

Conclusions: *C. parvum* was the main species found in all positive samples, with sequences belonging to only one subtype family, IIa (prevalent subtype, IIaA15G1R1) with high similarity of this subtype in calves to that in humans reported in Egypt and elsewhere. These finding highlight the importance of accurately defining *Cryptosporidium* genetic diversity throughout the country which helps in tracking infection sources in animals and humans, addressing the zoonotic potential role of calves in *Cryptosporidium* transmission to humans in Egypt. Also, significant hematological and biochemical alterations in infected calves can provide a good knowledge of the pathogenesis and thus help control the condition in the calf. The further studies must direct to study the role of the imported livestock in disease transmission, based on the high nucleotide homology with sequences from Latin America and European countries and the correlation of hemo-biochemical changes with the severity and progression of the Cryptosporidium infection.

Authors contribution: Each author contributed to the study's conception; AAS, and EBA contributed to the conception and design of the study. AAS, EBA and MMA conducted the laboratory works and drafted the manuscript. EBA, HG, and BME, analyzed the data and revised the paper. MBS, made data curation, editing and revised the manuscript. The final paper was read and approved by all authors.

Funding: For their research, writing, and/or publication of this work, the authors got no financial support.

Availability of data and materials: The corresponding author can provide the datasets used and/or analysed during the current investigation upon reasonable request.

Ethics approval: The research protocol was reviewed and approved by the ethics committee of the Faculty of Veterinary Medicine, Zagazig University, Egypt (approval number: ZU-IACUC/2/F/726/2022) and all procedures were carried out in accordance with the applicable rules and regulations. The study was carried out in accordance with ARRIVE guidelines.

Competing interests: There are no declared conflicts of interest for the authors.

REFERENCES

Abdou N, AlAzemi M and Al-Sayegh M, 2022. Performance of diagnostic assays used to detect Cryptosporidium oocysts in faecal samples of cattle in Kuwait and genotyping of Cryptosporidium species. BMC Vet Res 18:336.

Al-Laham N, Elyazji M, Al-Haddad R, *et al*., 2015. Possible hematological changes associated with acute gastroenteritis among Kindergarten children in Gaza. Annals Med Health Sci Res 5:292-298.

Amer S, Honma H, Ikarashi M, *et al*., 2010. Cryptosporidium genotypes and subtypes in dairy calves in Egypt. Vet Parasitol 169:382-386.

Brar A, Ahuja C, Sood N, *et al*., 2015. Hematological changes in neonatal diarrheic calves of different age groups. Indian J Vet Pathol 93:73-77.

Chen Y, Huang J, Qin H, *et al*., 2023. Cryptosporidium parvum and gp60 genotype prevalence in dairy calves worldwide: a systematic review and meta-analysis. Acta Tropica 240:106843.

Clustal W, Thompson JD, Higgins D, *et al*., 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 11:4673-80.

Constable P, 2004. Antimicrobial use in the treatment of calf diarrhea. J Vet Intern Med 18:8-17.

Constable P, Hinchcliff K, Done S, *et al*., 2017. Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats, 11th edition, Volumes 1 and 2. Can Vet J 58:1116.

Elmahallawy E, Sadek H, Aboelsoued D, *et al*., 2022. Parasitological, molecular, and epidemiological investigation of cryptosporidium infection among cattle and buffalo calves from Assiut Governorate, Upper Egypt: Current Status and Zoonotic Implications. Front Vet Sci 9:899854.

Essa S, Galila E, Abdelwahab M, *et al*., 2014. The incidence of Cryptosporidium infection among Friesian and buffalo calves in minufiya governorate. Benha Vet Med J 26:195-204.

Fayer R and Xiao L, 2007. Cryptosporidium and cryptosporidiosis. CRC press 1-42.

Felefel W, Abdel-Rady A, El-Rahim A, *et al*., 2023. Detection of Cryptosporidium parvum in calf feces using microscopical, serological, and molecular methods. Iraqi J Vet Sci 37:383-389.

Feltus D, Giddings C, Schneck B, *et al*., 2006. Evidence supporting zoonotic transmission of Cryptosporidium spp. in Wisconsin. J Clin Microbiol 44:4303-4308.

Gamsjäger L, Cirone K, Schluessel S, *et al*., 2023. Host innate immune responses and microbiome profile of neonatal calves challenged with Cryptosporidium parvum and the effect of bovine colostrum supplementation. Front Cell Infect Microbiol 13:1165312.

Gerrits W, Schrama J, Tamminga S, *et al*., 1998. The marginal efficiency of utilization of all ileal digestible indispensable amino acids for protein gain is lower than 30% in preruminant calves between 80 and 240 kg live weight. J Nutr 128:1774-1785.

Ghaffari S, Kalantari N and Charles A, 2014. Hart. A multi-locus study for detection of Cryptosporidium species isolated from calves population, Liverpool; UK. Int J Mol Cell Med 3:35.

Helmy Y, Krücken J, Nöckler K, *et al*., 2013. Molecular epidemiology of Cryptosporidium in livestock animals and humans in the Ismailia province of Egypt. Vet Parasitol 193:15-24.

Ibrahim M, Abdel-Ghany A, Abdel-Latef G, *et al*., 2016 "Epidemiology and public health significance of Cryptosporidium isolated from cattle, buffaloes, and humans in Egypt." Parasitol Res 115:2439-2448.

Iqbal A, Lim Y, Surin J, *et al*., 2012. High diversity of Cryptosporidium subgenotypes identified in Malaysian HIV/AIDS individuals targeting gp60 gene. PloS One 7:e31139.

Karanis P, Eiji T, Palomino L, *et al*., 2010. First description of Cryptosporidium bovis in Japan and diagnosis and genotyping of Cryptosporidium spp. in diarrheic pre-weaned calves in Hokkaido. Vet Parasitol 169:387-390.

Mahfouz M, Mira N and Amer S, 2014. Prevalence and genotyping of Cryptosporidium spp. in farm animals in Egypt. J Vet Med Sci 76:1569-1575.

Majeed Q, AlAzemi M, Al-Sayegh M, *et al*., 2022. Epidemiological and molecular study of cryptosporidium in preweaned calves in Kuwait. Animals 12:1805.

Muktar Y, Mamo G, Tesfaye B, *et al*., 2015. A review on major bacterial causes of calf diarrhea and its diagnostic method. J Vet Med Anim Health 7:173-185.

Mullakaev O, Zalaylov I, Kirillov E, *et al*., 2020. Morphological parameters of blood of calves with cryptosporidiosis after applying different treatment regimens. BIO Web of Conferences 17:00031

Naguib D, El-Gohary A, Mohamed A, *et al*., 2018. Age patterns of Cryptosporidium species and Giardia duodenalis in dairy calves in Egypt. Parasitol Int 67:736-741.

Panousis N, Siachos N, Kitkas G, *et al*., 2018. Hematology reference intervals for neonatal Holstein calves. Res Vet Sci 118:1-10.

Qi M, Zhang K, Huang M, *et al*., 2020. Longitudinal detection of Cryptosporidium spp. in 1–10-week-old dairy calves on a farm in Xinjiang, China. Parasitol Res 119:3839-3844.

Sahu B and Maiti S, 2011. Haematobiochemical Profile of crypotosporidiosis in Bovine Calves. Indian Vet J 88:63.

Saleh N, Allam T, Nayel M, *et al*., 2022. Hematological, Serum Biochemical and Parasitological investigation of calf diarrhea. Journal of Current Vet Res 4:58-68.

Song R, Kang J, Park K, *et al*., 2020. Analysis of hematological changes in normal and diarrhea calves. Korean J Vet Serv 43:161-165.

Sulaiman I, Hira P, Zhou L, *et al*., 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. J Clin Microbiol 43:2805-2809.

Taha S, Barghash S, Serag S *et al*., 2022. Prevalence and molecular characterization of Cryptosporidium sp. in ruminant livestock in South Sinai, Egypt. Bulletin of Faculty of Science, Zagazig University 2022:37-47.

Tamura K, 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+ C-content biases. Mol Biol Evol 9:678-687.

Thompson R and Ash A, 2016. Molecular epidemiology of Giardia and Cryptosporidium infections. Infect Genet Evol 40:315-323.

Tsukano K and Suzuki K, 2019."Plasma amino acid abnormalities in calves with diarrhea." Journal of Vet Med Sci 81:517-521.

Wang J, Diao Q, Tu Y, *et al*., 2012. The limiting sequence and proper ratio of lysine, methionine and threonine for calves fed milk replacers containing soy protein. Asian-Aust J Anim Sci 252:224.