Animal Health Research Journal Vol. 6, No. 2, June 2018 pp. 63-76

ISSN: 2356-7767

Coprological, serological and molecular diagnosis of *Toxocara* species in buffaloes, cattle and calves

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Received in 21/5/2018
Accepted in 27/6/2018

Abstract

In Egypt, *Toxocara vitulorum* (*T. vitulorum*) has a high prevalence rate in cattle and buffalo calves. The current work was designed to study the coprological, serological and phylogenetic characteristics of *T.vitulorum* in cattle, buffalo and calves in El Behera and Beni-suef governorates. Faecal samples were collected from 145 calves (89 buffaloes and 56 cattle calves) and were examined for the presence of *T. vitulorum* eggs by flotation and McMaster techniques. Blood and milk were collected from dams of the same animals (89 buffaloes and 56 cattle) and were examined for the presence of antibodies against *T. vitulorum* using two types of antigens (execratory /secretory (E/S) of infective stage larvae and adult worms) for serological diagnosis (ELISA). DNA was extracted from adult *Toxocara*. After PCR amplification, the sequencing of ND1 gene was carried out and phylogenetic analysis was performed using MEGA 6 soft ware. The result showed that 43(29.7%) calves were detected out to excrete *T.vitulorum* eggs in faeces. High number of *T.vitulorum* eggs were detected in faeces (63.8%) at > 6-9 weeks and the lowest number (6.6%) at 1-3 week. ELISA gave highest level of antibodies of *T.vitulorum* in serum and milk samples examined by E/S infective larval stages antigen than examined by E/S for adult worms antigen. Molecular analysis and phylogenetic tree of ND1 gene of *T. vitulorum* indicated that *T. vitulorum* Egy- strain has a common origin and genetically identical to those recorded in other countries.

Key words: *T. vitulorum, Faecal examination, Execretory/secretory antigen, ELISA, PCR, phylogenetic analysis*

Introduction

T. vitulorum is a nematode parasite of the small intestine of cattle and water buffaloes, particularly buffaloes calves between one and three months old, causing high morbidity and mortality (**Fabiano *et al.*, 2005**). The clinical signs of *T. vitulorum* infection are sometimes present, while many infections are subclinical (**Roberts 1993**). It is associated with diarrhea, poor performance, intestinal and biliary obstruction, and death (**Davila *et al.*, 2010**). This is accompanied by inappetence, intermittent colic, tympany, butyric odor in breath, loss of glossiness and skin tone with eczema signs resembling intestinal obstruction, presence of mud coloured foul smelling faeces, prominent ribs, and recumbence (**Li *et al.*, 2016**). It is clear from the life-cycle studies that mothers are the principal source of infection to calves through milk, therefore; detection of the parasite in immature larval stages in the dam is of paramount importance for control of toxocarosis in calves. Hence, for this purpose it is necessary to resort to immunodiagnostic techniques for early and accurate diagnosis. The use of milk for diagnosis and surveillance of

different diseases has become routine and milk antibody testing plays a significant role in cattle disease control and eradication programs in many countries. It has been shown for many infections that there is generally a good qualitatively between milk and serum antibody titers, but milk sampling is easier, cheaper and non-invasive compared to blood sampling (**Pritchard *et al.,* 2002**). The larvae remain in the tissues of the infected dam until just prior to parturition then migrate to the mammary gland and milk for subsequent ingestion by the calf (**Starke-Buzetti and Ferreira, 2006**).This parasite is acquired vertically by calves when they suckle milk contaminated with infective larvae (**Starke *et al.,* 1992**). The newly born calves were found to be highly infected at age of 15-90 days and consequently act as a source of infection to other animals in the farm as infected calves started to shed eggs at 16-23 days of age (**Raza *et al.,* 2013**). *T. vitulorum* infection can be diagnosed on the basis of clinical signs, necropsy finding, faecal examination for eggs and serological tests. Coprological examination techniques fail to detect infections in adults, also during the pre-patent stage and in mild infections in young calves (**Pritchard *et al.,* 2002**). ELISA is specific and very sensitive in detection of infection as early as 2 weeks post infection (**Jin *et al.,* 2013)**. Anti-*T. vitulorum* antibodies were detected in the milk samples from infected buffaloes by ELISA using adult and execretory secretory antigens of larval stages (**Hassan and Abdel Aziz, 2010**). Various antigens like soluble extract and excretory/secretory antigen from *T. vitulorum* larvae and adults, perienteric fluid antigen of adult parasite and crude antigen of adult *T. vitulorum* have been used by several workers (**De Souza *et al 2004,* Ferreira and Starke 2005**). Antibodies against larval excretory / secretory (ES) and larval soluble extract (EX) of *T. vitulorum* were detected in serum of buffalo cows and calves naturally infected with *T. vitulorum* , indicating that *T.vitulorum* infection can stimulate the immune system of the buffalo (**Starke-Buzetti *et al.,* 2001**). Colostrum antibody concentration was highest on the first day post-parturition, but decreased sharply during the first 15 days. Anti *T. vitulorum* antibodies were detected in the colostral samples from infected buffaloes by ELISA using somatic and execretory / secretory antigens (**Ghosh and Banerja 1998**). Current DNA techniques are used widely to identify parasite species more precisely to assess the genetic diversity among parasite populations (**Mochizuki *et al.,* 2006**), and to determine the phylogenetic relationships among the parasitic species (**Nadler and Hudspeth, 1998**). The current study was designed to devote throw light on toxocariasis among buffaloes and cattle in El-Behera and Beni-Suef governorates. In addition to evaluate the ELISA by using two types of antigens (excretory-secretory antigens of larvae and adult) for diagnosis of *Toxocara* in serum and milk and phylogeny of *T. vitulorum* using PCR sequencing of a mitochondrial gene of ND1.

Materials and Methods

Animals and Samples:

Faecal samples: Faecal samples were collected from buffalo (89) and cattle (56) calves (1- 20 weeks) from different farms at El-Behera and Beni-Suef governorates. These samples were collected from the animals in sterile disposable plastic bags that were closed tightly and labeled with their age, place and date of collection and clinical status of animals. These samples were classified according to the following schedule: from 1 - 3 weeks, >3 - 6 weeks, >6 -9 weeks and >9- 20 weeks. Samples were sent to laboratory as quick as possible for investigation.

Collection of parasites: For preparation of two types of antigens (E/S adults and infective larval stages) and PCR, adult *T. vitulorum* (males and females) were collected from buffalo calves found heavily positive for *T. vitulorum* eggs using faecal flotation method Fig. (1). The positive animals were administrated piperazine (100mg/kg) orally once, followed by liquid paraffin (15-20 ml) after 8-12 h inter-

val to increase the intestinal motility and to facilitate the worm expulsion. After 24- 36h, *T. vitulorum* parasite were expelled with the faeces from animals (**Jyoti *et al.,* 2011**). These collected parasites were washed immediately with distilled water and normal saline solution repeatedly to remove the mucus and entangled excreta and then identified through morphological examinations. For PCR technique, total genomic DNA of parasite extraction was performed employing DNA extraction reagent kit (TIANamp Genomic DNA Kit, Tiangen Biotech Co., Beijing, China) according to manufacturer's recommendations. The eluted DNA was stored at -20˚C prior to PCR analysis (**Li *et al.,* 2016**).

Blood and milk samples: Blood and milk samples were collected from dams of the same buffaloes and cattle calves after parturition. Serum samples centrifuged at 3000 rpm for 15 minutes at 4℃. The samples of colostrum were centrifuged at 4°C in refrigerated centrifuge at 3000 rpm for 10 minutes. After removal of solidified fat, the samples were left in an incubator at 37°C for 1 hour for casein precipitation with one percent rennin. Then the milk serum was separated by centrifugation for 15 minutes at 3000 rpm at 4°C (**Starake-Buzetti, 2006**). Positive control serum was collected from heavily infected buffalo calves with *T. vitulorum*, as diagnosed by faecal examination. Negative control sera collected from healthy buffalo calves (at one age before suckling the colostrum and negative by faecal examination). Serum and milk samples were separated, aliquated and stored at – 20°C.

Microscopical examination:

All collected faecal samples (89 buffaloes and 56 cattle calves) were examined by using flotation technique to detect *Toxocara* eggs. Eggs counted by McMaster technique according to **Soulsbay (1986**).

Serological examination:

Preparation of excretory-secretory (E/S) antigen of adult *T. vitulorum* :

The excretory-secretory products (E/S) were prepared according to **River Marrero *et al***., (**1988**) as follows: *T. vitulorum* adult worms (males and females) washed repeatedly (3-5 times) in 0.01 M PBS, pH7.4 supplemented with antibiotic. The worms were then incubated in 0.01 M PBS, pH 7.4 (one worm /5ml) for 3 hours at 37ºC. After incubation, the worms were removed and the fluid (PBS+E/S) was collected and subjected to high speed centrifugation (12000rpm) for one hour at 4ºC. The protein content was measured by the method of **Lowry *et al.,* (1951**). The antigen was aliquoted and stored at – 20℃ until use.

Preparation of excretory-secretory antigen of *T. vitulorum* infective larvae according to Hayat *et al.,* (1997):

Ten mature Toxocara females were dissected and the uteri extruded and transferred to a pestle and mortar in which 5 gms coarse sand was added. The mixture was ground gently and 2% formalin was added. The mixture was then centrifuged and supernatant was collected containing *T. vitulorum* eggs. The supernatant along with 2% formalin was incubated at 28°C for 14-18 days. Emberyonated eggs were suspended in PBS and ultrahomogenized for 5 minutes at 4°C. The suspension was centrifuged and the collected supernatant was used as an antigen. The protein content was measured by the method of **Lowry *et al.,* (1951**). The antigen was aliquoted and stored at – 20ºC until use.

Enzyme-Linked immunosorbent assay (ELISA):

Specific antibody against *Toxocara* antigens (execratory /secretory of adult and larvae) was detected in buffaloes and cattle tested sera or milk by ELISA according to **Zimmerman *et al.***, (**1982**). Optimal serum or milk antigen, and bovine anti- IgG peroxidase conjugate concentrations were determined after preliminary checkerboard titration. While, defatted skim milk samples were utilized without dilution, wells in ELISA plates were coated with 100 ul of different Toxocara antigens of coated buffer overnight at 4°C. After washing with PBS, wells were saturated with 100ul/well of PBS containing 5% BSA for 1 hour. 100 µl serum

diluted 1:100 in PBS-T was dispensed as duplicate to each well after washing and incubated for 1h. Plates were washed and then 100µl of anti-bovine IgG horseradish peroxidase conjugate in 1:5,000 dilution prepared in 1% skimmed milk in PBS was added to each well and incubated at 37°C for 1 hour. After washing 40mg O-phenylendiamine dihydrochloride (OPD in100ml of phosphate citrate buffer, pH 5.0 and 40µl H2O2 was added to each well and kept in dark for color development. The reaction was stopped after 2min using50µL of 3N HCl in each well. The optical density (O.D) was measured at 450 nm for paired samples against blank control well. The tested sera were considered to be positive when the absorbance values were as more than the cut off values (**Allan *et al.*, 1992**).

Gene amplification and DNA electrophoresis (Li *et al.*, 2016):

PCR amplification approach was used to amplified a fragment (-370 bp) of the NADH dehydrogenase subunit 1 (ND1) of mitochondrial (mt) gene. During the PCR, the primer pairs (forward: 5′-TTCTTATGAGATTGCTTTT-3′ and reverse: 5′-TATCATAACGAAAACGAGG-3′) were used. The PCR mixture contained 9.75 µl autoclaved, distilled water, 5 µl PCR buffer (10×), 3 µl MgCl2 (25 mM), 2 µl dNTPs (2.0 mM), 1 µl DNA, 0.25 µl Taq, 2 µl of each forward and reverse primer (working concentration: 10 µmol/L) in a 25 µl reaction volume. Each of the 40 PCR cycles consisted of 94ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 1 min after an initial hot start at 94ºC for 5 min and ending with 72ºC for 5 min. PCR products were analyzed on a 1% agarose gel stained with ethidium bromide following electrophoresis. The PCR electrophoresis products were purified using a Hi-TIANgel Midi Purification Kit (Tiangen Biotech Co., Beijing, China) according to manufacturer's recommendations.

Molecular cloning and sequencing of the ND1 gene (Li *et al.*, 2016):

PCR products were purified using QIAquick PCR product extraction kit (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column (Elim biopharm centicals, Germany). DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul *et al.*, 1990)** was initially performed to establish sequence identity to Gen Bank accessions. The phylogenetic tree was created by the MegAlign module of Lasergene DNA Star **Thompson *et al.*, (1994)** and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 **(Tamura *et al.*, 2013).** Sequence data were compared with other ascarids strains available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These strains were *T. vitulorum, Toxocara canis, Ascaris lumbricoides, Ascaris suum, Anisakis simplex, Ascaridia galli, Pseudoterranova azarasi, Parastrongyloides trichosuri, Angiostrongylus cantonensis* and *Steinernema kushidai.*

Results

Microscopical examination: Forty three out of 145 (29.7%) faecal calve samples (89 buffaloe and 56 cattle calves) found to be positive by flotation techniques for *Toxocara* as shown in table (1). The *Toxocara* eggs were detected with a percentage of 6.6%, 40%, 63.8 % and 9.1% in calves at 1- 3, >3-6, > 6-9 and > 9-20 weeks respectively. High number of *T. vitulorum* eggs was detected in faeces of calves at > 6-9 weeks (63.8%) and low number (6.6%) at 1-3 weeks. The eggs of *Toxocara* appeared as subglobular, provided with a finely pitted albuminus layer and measured 68-85 by 58-72 µm. Fig. (2).

Table (1). Results of coprological examination of *Toxocara* infection in buffaloes and cattle calves according to age:

Age of buffalo and cattle calves	No. of examined animals	No. of positive animals	% of infection
1- 3 weeks	30	2	6.6 %
>3- 6 weeks	35	14	40 %
>6- 9 weeks	36	23	63.8 %
>9- 20 weeks	44	4	9.1 %
Total	145	43	29.7 %

Serological results:

ELISA was carried out on 89 sera of buffaloes and 56 of cattle to detect *Toxocara* antibodies against the two types of antigens (E /S for infective stage larvae and adult worms). In buffaloes, 34 (38.2%) serum samples were positive to *Toxocara* antibodies against E/S antigen for larval stages while, 29 (32.6%) samples were positive against E/S for adult *Toxocara* antigen. In cattle, fifteen (26.8%) serum samples were positive to *Toxocara* antibodies against E/S infective stage larvae antigen, while 10 (17.9%) samples were positive against *Toxocara* E/S adult antigen. The present study showed that serological results (ELISA) of milk samples collected from buffaloes revealed that the prevalence of *Toxocara* infection were 26/89 (29.2%) and 24/89 (27%) by using E/S antigens of larvae and adults respectively. While, in cattle 13/56 (23.2%) and 10/56 (17.9%) were positive by using E/S antigens of larvae and adults respectively (Table 3). In the present study, highest level of antibodies of *T. vitulorum* in all serum and milk samples examined during the perinatal period by E/S larval antigen than examined by E/S for adult antigen.

Table (3). ELISA results of *Toxocara* antibodies in serum and milk samples of buffaloes and cattle:

Examined animals	Serum samples		Milk samples	
	E/S antigen of infective stage larvae	E/S antigen of adult warms	E/S antigen of infective stage larvae	E/S antigen of adult warms
Buffaloes (89)	34 (38.2%)	29 (32.6%)	26 (29.2 %)	24 (27%)
Cattle (56)	15 (26.8%)	10 (17.9%)	13 (23.2 %)	10 (17.9%)
Total (145)	49 (33.8%)	39(26.9%)	39 (26.9 %)	34 (23.4%)

Molecular results:

DNA was successfully PCR amplified, fragments (-370bp) from female adult of *T. vitulorum* (Fig. 3). Alignments of our representative profiles of ND1 sequences of the current parasite with other ascarids available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the percentage of nucleotide identity was highest with *Toxocara vitulorum* H13 (KY825181.1) and *Toxocara vitulorum* H1 (KY825180.1) with a percentage of 94.6%, *Toxocara vitulorum* Tvit3-1(AJ937266.1) with a percentage of 94.1% , and *Toxocara vitulorum* (FJ664617.1) with a percentage of 93.8%. These percentages ranging from 86.7 to 85.3 with *Toxocara canis* T can13 (AJ920385.1), *Toxocara canis* T. can10 (KC293922.1), *Toxocara canis* Tcat2-2 (AJ920387.1), *Toxocara canis* Tcan8-1 (AJ920383.1), *Toxocara canis* T.can7(KC293920.1, *Toxocara canis* (AM41110.8.1), *Toxocara canis* (EU730761.1), *Ascaris lumbricoides* G17 (KY04502.1), *Ascaris sum* PTZ2 3 (KY045800.1) and *Ascaris sum* (HQ70490.1) respectively (Fig. 4). Sequence distance generated by MegAlign module of Lasergene

DNAStar showing maximum identity range of 93.8-94.6% between Egyptian strain and international *T. vitulorum* strains uploaded from genebank (5). The phylogenetic analysis (Fig.6) revealed that the Egy strain has a common origin with *Toxocara vitulorum* H13, *Toxocara vitulorum* H1, *Toxocara vitulorum* Tvit3-1and *Toxocara vitulorum*, with a percentage of 94.6%, 94.6%, 94.1% and 93.8% respectively. Table (4) showed source of isolates used for comparison with the current isolate (Egy- strain) at the molecular level.

Fig. (1): Adult female worms of *Toxocara* (18.5 – 27 cm. long)

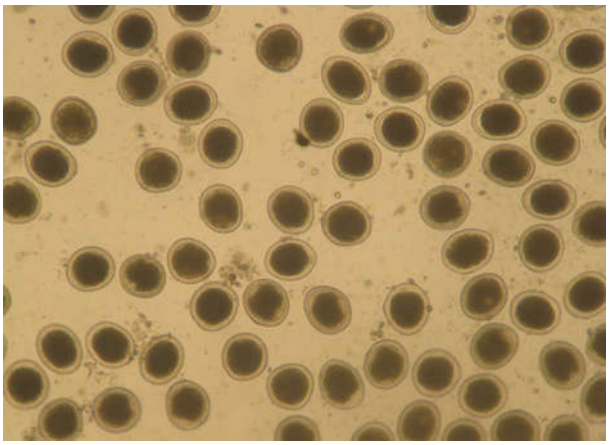

Fig. (2): Eggs of *Toxocara* in faecal samples using flotation technique X40

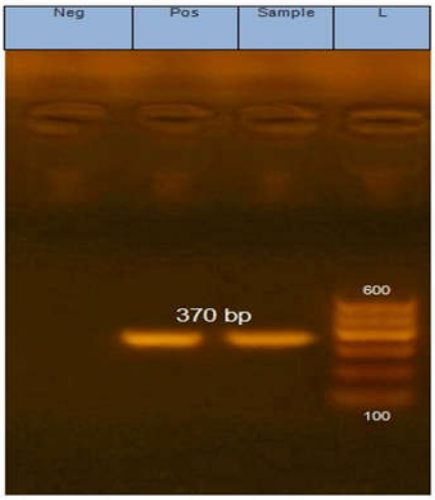

Fig. (3):
PCR amplification of ND1 gene fragment from *T. vitulorum*. PCR positive samples for *T. vitulorum* (-370 bp).
Lane (L): Marker from 100-600
Control positive and negative samples.

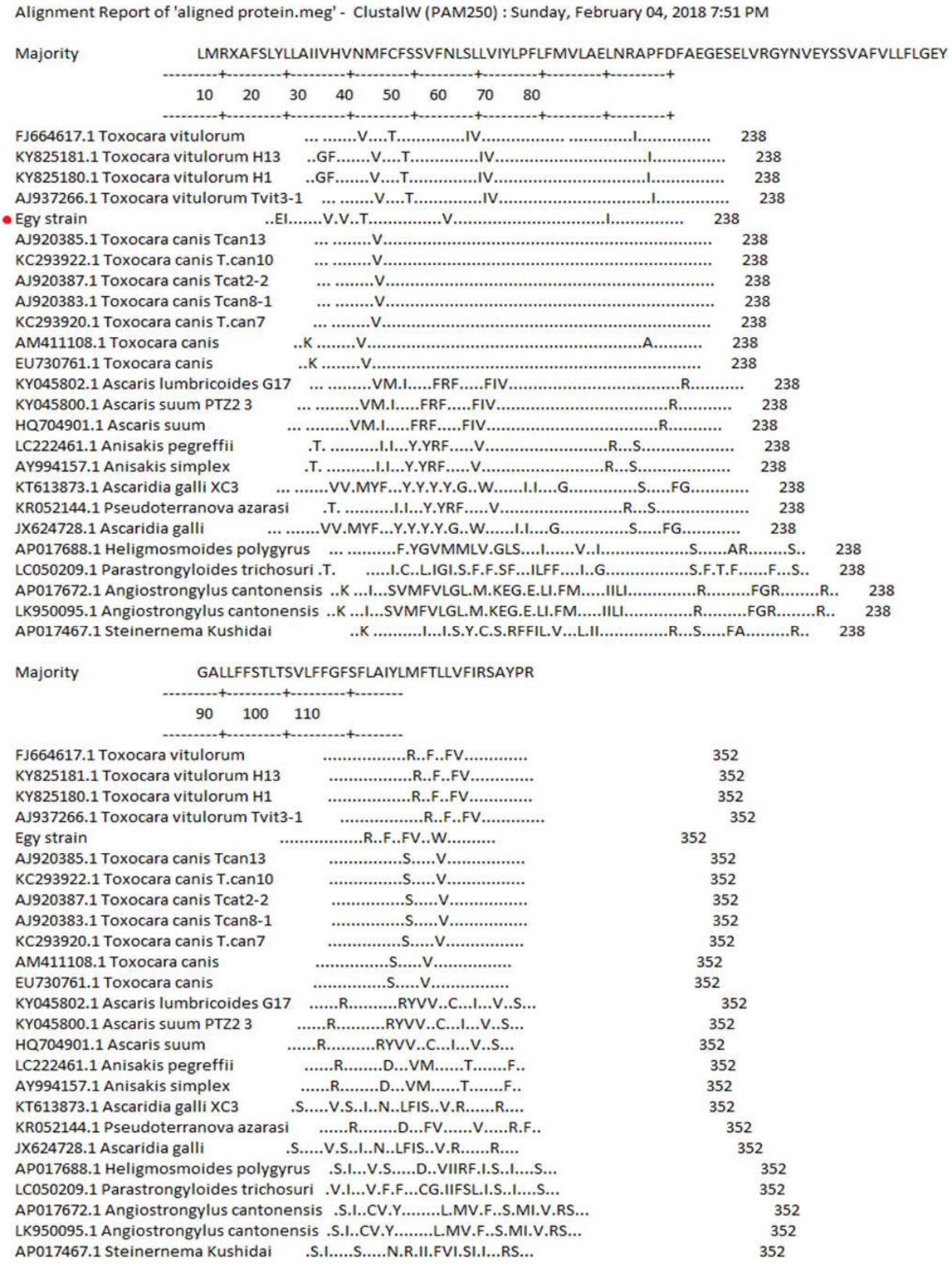

Fig. (4): Amino acid alignments report for Egy-strain of *T. vitulorum* and representative Ascarides for ND1 gene.

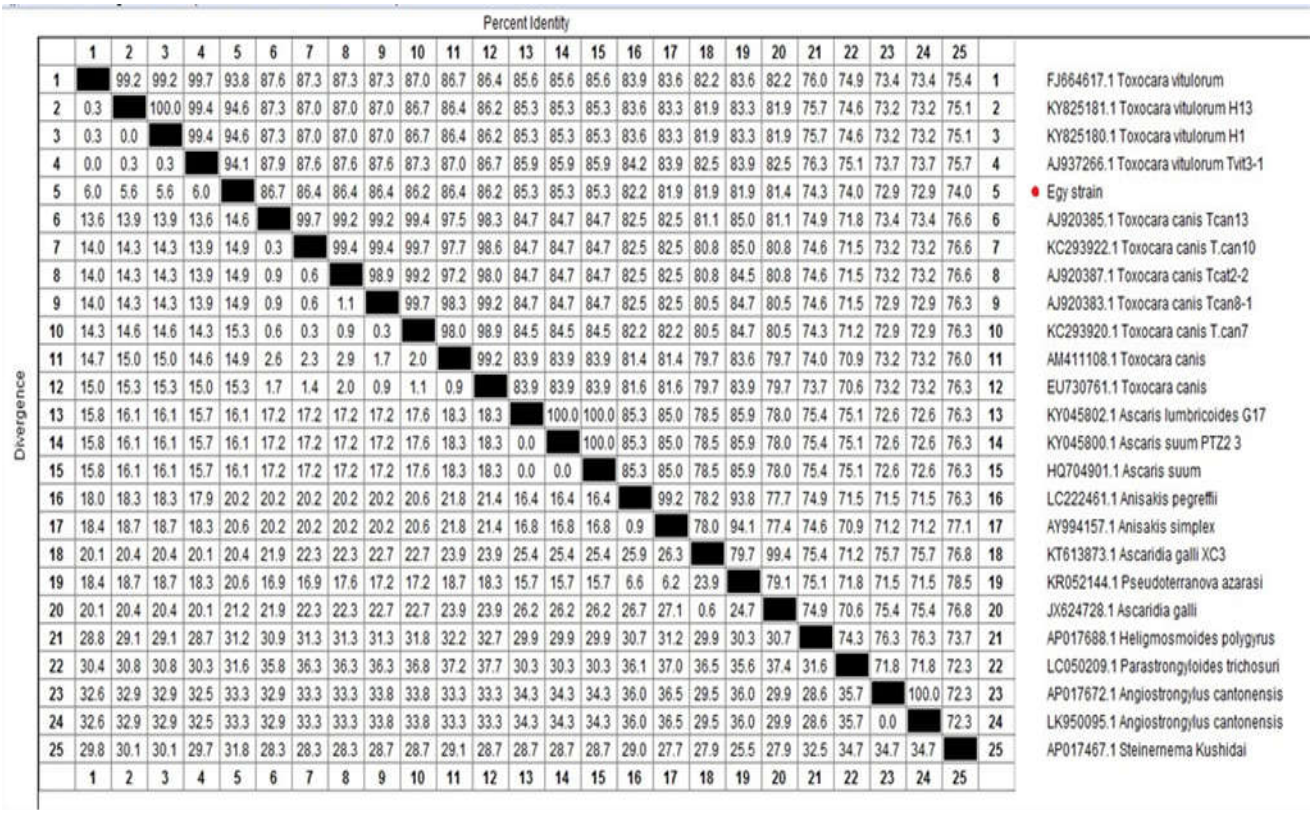

Fig. (5): Sequence distance generated by Meg Align module of Laser gene DNA Star showing maximum identity range of 93.8-94.6% between Egy- strain and international *T. vitulorum* strains uploaded from Gene Bank.

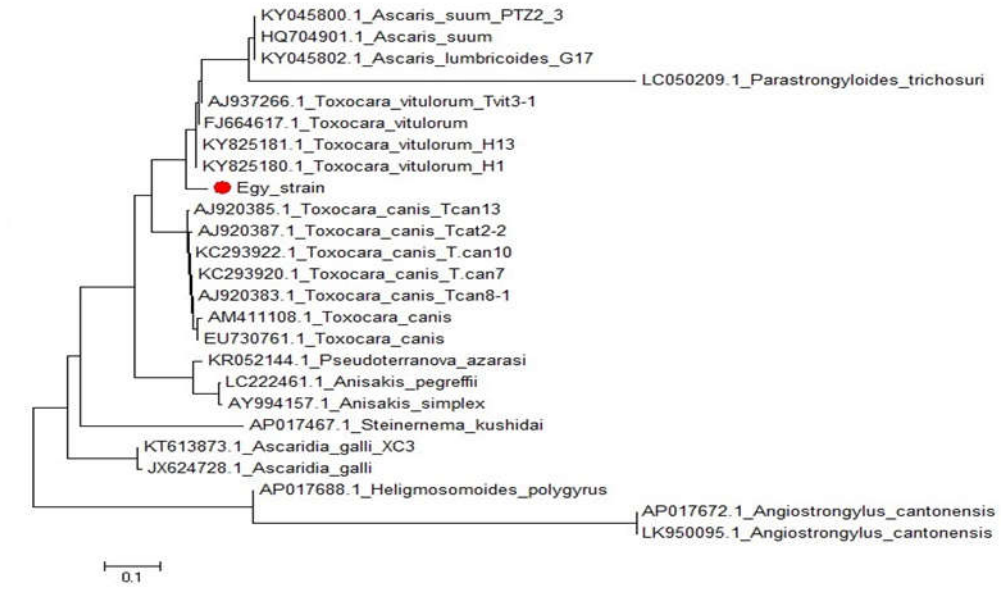

Fig. (6): Phylogenetic tree illustrating the origin of the present isolate of *T.vitulorum* (Egy- strain) with other isolates.

Table (4). Table illustrating the source of isolates used for comparison with the present isolate (Egy- strain) at the molecular level.

Parasite accession number in Gene Bank	Source	Parasite accession number in Gene Bank	Source
Ky045800 ascaris suum ptz2-3	DENMARK	Eu730761.1 toxocara canis	AUSTRALIA
Hq704901.1 ascaris suum	SOUTH KOREA	Kr052144.1 pseuterranova azarasis	CHINA
Ky045802.1 ascaris lumbricoides g17	DENMARK	Lc222461.1 anisakis pegriffii	JAPAN
Aj937266.1 toxocara vitulorum tvit3-1	SERILANKA	Ay994157.1 anisakis simplex	KOREA
Fj664617.1 toxocara vitulorum	JAPAN	AP017467.1 STEINERNEMA KUSHIDAI	JAPAN
Ky825181.1 toxocara vitulorum h13	JAPAN	Kt613873.1 ASCARIDIA GALLI-XC3	CHINA
Hy825180.1 toxocara vvitulorum h1	JAPAN	JX624728.1 ASCARIDIA-GALLI	CHINA
Aj920385.1 toxocara canis t can 13	AUSTRALIA	AP017688.1 HELIGMOSOMOIDES POLYGYRUS	JAPAN
Aj920387.1 toxocara canis t cat 2-2	CHINA	AP017672.1 ANGIOSTRONGYLUS CANTONESIS	JAPAN
Kc293922.1 toxocara canis t can 10	IRAN	LK950095.1 ANGIOSTRONGYLUS CANTONESIS	TAIWAN
Kc293920.1 toxocara canis t can 7	IRAN	Lc050209.1 Parastrongyloides trichosuri	japan
Aj920383.1 toxocara canis t can 8-1	AUSTRALIA		
Am411108.1 toxocara canis	CHINA		

Discussion

T. vitulorum, a parasite of the small intestine of cattle and buffaloes, is mainly acquired by calves via milk from infected cows. Parasitic status of the calves for *T. vitulorum* infection was evaluated by sequentially fecal examination from one to 20 weeks of age. Absence of eggs in the faeces does not mean that animals free from infection. In the present study, 43 out of 145 (29.7%) faecal samples found to be positive by flotation techniques for *Toxocara*. The *Toxocara* eggs were detected with a percentage of 6.6%, 40%, 63.8 % and 9.1% calves at 1- 3, > 3-6, > 6-9 and > 9-20 weeks respectively. *T. vitulorum* eggs in faeces of calves reached to the peak number (63.8%) at >6-9 weeks. In India, **Yadav *et al.,* (2004)** and **Bhutto *et al.,* (2002)** reported the percentages of infection in buffalo calves were 27.58% and 32% respectively. Meanwhile, **Afridi *et al.,* (2007)** in Pakestan and **Halmandge *et al.,* (2005)** in Bidar found the percentages of infection in buffalo's calves with *T. vitulorum* were13.14% and 6% respectively. **Singh *et al.,* (2008)** and **Muraledharam (2005)** found the infection of *T. vitulorum* in water bufalloe and cattle calves were 1.28%, 7.75% and 5.44%, 0.14% respectively. On the other hand, **Kumari *et al.,* (2004)**, **Kaur and Kaur (2008)** and ***Li et al .,*(2016)** reported a higher rate of infection in water buffaloes with toxocariasis were 41.66%, 38% and 64% but in cattle were 47.21% and 78.57% and 75% respectively. (**Boes *et al.,* 1997**) showed that the coprological diagnosis of *T. vitulorum* infection is not accurate because the positive diagnosis by this method requires mature worms producing eggs, extensive hepatopulmonary larval migration with resulted pathological changes have occurred before such diagnosis is possible. Early diagnosis via immunological techniques would allow initiation of therapy before extensive larval migration and resultant pathologic changes. Accuracy of immunodiagnosis de-

pends on the development of satisfactory extract and successful choice of the assay. The selection of ELISA in the present research was based on previous studies suggesting ELISA as a good tool for serodiagnosis of toxocariasis (**Wickramasinghe *et al.,* 2009**). In the present study, serological diagnosis by ELISA in 89 buffaloes calves revealed that 38.2 % and 32.6 % serum samples were positive to *Toxocara* antibodies against E/S of infective stage larvae and adult worms antigens respectively. In 56 cattle, 26.8% and 17.9 % serum samples were positive to *Toxocara* antibodies against E/S of infective stage larvae and adult worm antigens respectively. The present study showed that serological results (ELISA) of milk samples collected from the same animals revealed that the prevalence of *Toxocara* infection were 29.2% , 27% , 23.2% and 17.9% in cattle using E/S of infective stage larvae and adult worm antigens respectively. In the present work, the level of antibodies of *T.vitulorum* in all serum and milk samples examined during the perinatal period by E/S of infective stage larvae antigen were higher than that examined by E/S for adult antigen. The presence of antibodies in larval E/S in the serum of buffalo cows can be related to larval infection; the infection in the cows can be confirmed indirectly because calves only acquire infection with adult *T. vitulorum* from milk (**Roberts *et al.,* 1990**). Since the cow and its calf were penned with each other for the first week, which is the main period for the transmission of larvae from colostrum/milk essentially all the adult *T. vitulorum* acquired by each calf would have originated from its own dam (**Amerasinghe *et al.,* 1994**). In Sri Lanka, **Rajapakse (1994) and Wijesundera *et al.,* (2003**) used antigens of infective stage larvae for detection of antibodies against *T. vitulorum* using ELISA. In Egypt, **Abdel-Rahman** and **Ashmawy (2013**) reported higher results (63.4%) using serological diagnosis (indirect ELISA). They added that perienteric fluid antigen (Pe) of *T. vitulorum* was used to monitor the humeral immune – response by an indirect ELISA in the infected pregnant buffaloes and their newly born calves. Also, they reported the *T. vitulorum* were diagnosed in milk of infected lactating buffaloes specially in the first few days post parturition then decreased sharply within 15 days. In Egypt, **Hassan and Abdel Aziz** (**2010**) applied the ELISA test for studying the *T. vitulorum* in buffalo's calves using somatic and execretory- secretory antigens. They found the execretory- secretory antigen was the most potent one for diagnosis of toxocariasis in buffalo calves by a percentage of 73.8%. **Mahdy *et al.,* (2017)** used different *T. vitulorum* antigens; (E/S antigen, peri-enteric fluid antigen and embryonated eggs antigen) for evaluation of induction of protection against a chalange of oral infection with embryonated eggs of *T. vitulorum* using ELISA). **Starke- Buzetti *et al*.**, (**2001**) used indirect ELISA for the detection of antibodies against *T. vitulorum* in sera of 15 water buffaloes after birth and 9 buffalo cows during the perinatal period using soluble extract antigen. They found the antibody level was lowest in all serum samples examined during the first days and highest in calves at 1 day of age before and after suckling colostrum. **De Sousa *et al*.**, (**2004**) detect antibodies of *T. vitulorum* in the serum and colostrum of buffaloes cows and calves naturally infected animals. They added that the highest level of antibodies against perienteric antigen (Pe) of *T. vitoulourum* were detected in buffalo cow sera during the perinatal period and were maintained in high level through 300 days after parturition. They concluded that ES antigen was the most potent in immunodiagnosis of toxocariasis. **Ghosh and Banerjee**, (**1998**) detect the antibodies of *T. vitoulorum* in serum and colostrum samples from naturally infected buffaloes by ELISA using somatic and excretory secretory antigens and concluded that the ES antigen is the most potent antigen. **Souza** (**2001**) showed that the highest level of anti-Ex antibodies of *T.vitulorum* were detected by ELISA in buffalo cow sera during the perinatal period and were maintained at high levels through 300 days after parturition. Sequence

analysis of the current parasite with other ascarids revealed that the percentage of nucleotide identity was highest with *Toxocara vitulorum* H13 and *Toxocara vitulorum* H1 with a percentage of 94.6%, *Toxocara vitulorum* Tvit3-1 with a percentage of 94.1%, and *Toxocara vitulorum* with a percentage of 93.8%. These percentages ranging from 86.7% to 85.3% with *Toxocara canis* T. can13, *Toxocara canis* T. can10, *Toxocara canis* Tcat2-2, *Toxocara canis* Tcan8-1, *Toxocara canis* T.can7, *Toxocara canis, Toxocara canis* , *Ascaris lumbricoides* G17, *Ascaris sum* PTZ2 3 and *Ascaris sum* respectively. The phylogenetic analysis revealed that our isolate (Egy-strain) has a common origin with *Toxocara vitulorum* H13, *Toxocara vitulorum* H1,*Toxocara vitulorum* Tvit3-1 and *Toxocara vitulorum*, with a percentage of 94.6% , 94.6%, 94.1% and 93.8% respectively. Several genomic regions have been used for phylogenetic studies of different parasite species including 18S and 28S ribosomal RNA as well as mitochondrial genes of Cox1 and ND1 (**Nagataki *et al.,* 2015**). **Wickramasinghe *et al.,* (2009)** determined the phylogenetic position and genetic diversity within the genus *Toxocara* using two mitochondrial genes (comlete ATPase 6 and partial small subunit ribosomal RNA (12S rDNA)), two nuclear ribosomal genes (second internal transcribed spacer region (ITS -2)) and part of the large subunit 28S region. Nucleotide sequence (597bp) and predicated amino acid sequences of the complete ATPase 6 genes (199 amino acids) of both species (*T. canis* and *T. vitulorum*) are similar in size with the *Toxocara cati* and *Toxocara malaysiensis*. They added that there was 88% nucleotide similarity between *T. canis* and *T. vitulorum* and many transversions present in the 12S gene. **Sultan *et al.,* (2015)** studied the molecular and phylogenetic tree of *T. vitulorum* isolated from cattle in the mid- Delta of Egypt using Internal Transcribed Spacer (ITS-1) and 18S genes of ribosomal RNA. They concluded that *T. vitulorum* isolated from cattle in Egypt is genetically identical to other Toxocara species and the phylogenetic trees show a close relationship among these different species of *Toxocara* including the zoonotic species. **Li *et al*.**, (**2016**) studied the phylogenetic characteristics of *T. vitulorum* in different areas in China using ND1 gene. They found that the *T. vitulorum* isolates in the different areas were homologous and this kind of ascariasis was a local endemic disease.

In conclusion, this study confirms that *T. vitulorum* isolated (EGY-strain) in Egypt is genetically identical to other *Toxocara* species and the phylogenetic trees show a close relationship among these different species of *Toxocara* including the zoonotic species.

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