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PCR and Serological Assays for Detection of *Toxoplasma gondii* Infection in Sport Horses in Cairo, Egypt

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ABSTRACT

A total of 240 serum samples collected from horses of different ages, breeds, sexes and reproductive conditions used for sporting purposes and located at main horse farms in Cairo, Egypt, were tested for *Toxoplasma gondii* infection. PCR and serological assays revealed that, PCR showed the higher prevalence of toxoplasmosis (53.8%) followed by LAT (52.1%), MAT (50.8%) and lowest prevalence by ELISA (39.2%). Prevalence of *T. gondii* infection in relation to breed, sex and reproductive condition was determined. Prevalence was higher (73.1%) in imported breeds followed by native (58.5%) and lowest prevalence in Arabian breeds (44.4%). Higher prevalence (60.8%) was detected in females represented by 10.8, 12.4 and 29.9% in fillies, pregnant and repeat breeders, respectively. On other hand, lower prevalence (23.9%) was detected in males represented by 0, 8.7 and 15.2% in colts, stallions and racers, respectively. When the data of the serological tests were compared with that of the PCR, as a reference test for toxoplasmosis, MAT had the highest sensitivity (93.8%) followed by LAT (91.5%) and the lowest sensitivity by ELISA (71.3%). On the other hand ELISA had the highest specificity (92.8%) followed by MAT (91.9%) and the lowest specificity was by LAT (88.3%). The present study is the first time to adopt PCR and serological survey of *T. gondii* antibodies in sport horses in Egypt and suggests that MAT alone or with LAT can be used as a highly sensitive screening test followed by PCR as a specific confirmatory test for diagnosis of toxoplasmosis in Equines. Also, the high prevalence observed indicate that the risk of infection from horses to people or other animals is very high.

Key words: *Toxoplasma gondii*, sport horses, PCR, LAT, MAT and ELISA

INTRODUCTION

All hosts, including humans can be infected by any one of the three forms of the *Toxoplasma gondii* parasite that correspond to three morphological stages: Tachyzoite, bradyzoite and sporozoite form. Felids are definitive hosts for *T. gondii*, which is an intracellular pathogen that infects a wide range of warm-blooded intermediate hosts. Toxoplasmosis is a disease where the interests of the diverse medical and veterinary specialties converge (Cenci-Goga *et al.*, 2011). *T. gondii* infection does not cause serious illness but in pregnant women and female animals it is a common cause of abortion, blindness, megallocephally and mental retardation to children. Whereas animal fetuses were mummified, macerated and stillborn or may be borne weak and die within weeks after birth and the parasite also, emerged as a major cause of death in patients with acquired immunodeficiency syndrome (Innes, 2010).

Although, viable *T. gondii* has been isolated from horses slaughtered in Zoo abattoir, horse meat is not used for human consumption in the Egypt (Shaapan and Ghazy, 2007) and the *T. gondii* cysts has been shown in edible tissues from horses (Al-Khalidi and Dubey, 1979). The role of horses as a source of *T. gondii* infection depends on regional preferences for horse meat, the preparation method and the seroprevalence of horses used for consumption raw or undercooked in countries such as Belgium, Italy, France and Japan (Gill, 2005).

Few data about the prevalence *T. gondii* infection in sport horses has been reported in some countries, the prevalence was 28% in Turkey horses bred for sportive purposes in the province of Ankara using the Sabin-Feldman Dye Test (DT) (Guclu *et al.*, 2007), 71.2% in Iran using MAT (Hajjalilo *et al.*, 2010) and 43.7% in horses for sporting purposes in the Province of Riyadh, Saudi Arabia using DT (Alanazi and Alyousif, 2011).

The prevalence data for the determination of *T. gondii* infection in horses in the different localities of the world is extremely variable and based mainly on different serological tests. The prevalence was 97.1% in Argentina using Indirect Haem-Agglutination Test (IHAT) (Mayer *et al.*, 1987), 1% in Swedish horses using ELISA (Uggla *et al.*, 1990), 6.9% in North America using MAT (Dubey *et al.*, 1999), 23% in Czech Republic using LAT (Bartova *et al.*, 2010) and 34% in Costa Rica using ELISA (Dangoudoubiyam *et al.*, 2011).

In Egypt, although there is no data about *T. gondii* prevalence in sport horses, there are a little known investigation about the prevalence of the infection in equines. In horses, the *T. gondii* antibodies was found in 38.1 and 48.1% using ELISA and MAT, respectively (Ghazy *et al.*, 2007), while Haridy *et al.* (2009), reported that the overall ELISA-*T. gondii* antibodies were 25% of the horses in Greater Cairo, 50 (females) and 22.2% (males). In donkeys, the infection rate was 65.6% using ELISA (El-Ghaysh, 1998) and recently, Shaapan and Khalil (2008) recorded that prevalence of *T. gondii* antibodies in donkeys was ranged from 36 to 52% using the different *T. gondii* isolates as antigens of MAT.

The present work was aiming to studying the prevalence of *T. gondii* infection among sport horses located in some farms in Cairo using PCR and serological assays (LAT, MAT and ELISA). The prevalence of infection in relation to breed, sex and reproductive status was investigated. Moreover, sensitivity, specificity, advantages and disadvantages of each test were also determined.

MATERIALS AND METHODS

Blood and serum samples: A total of 240 blood samples were collected by venipuncture from sport horses located at some horse farms in Cairo, Egypt. Data regarding these animals (age, gender, races, breeds and reproductive conditions) were also collected. Sera were separated, labeled and stored at -20°C until used for serological and PCR testing.

Strains of *T. gondii*

***T. gondii* RH strain:** Virulent RH strain of *T. gondii* used for PCR was obtained from colony maintained in Department of Zoonoses, National Research Center.

Local isolated *T. gondii* strain: *T. gondii* local strain used for antigen preparation was successfully obtained by Shaapan and Ghazy (2007), after many trails of bioassay of the suspected infected horse tissues in cat and mice as the procedures described by Sharma and Dubey (1981) and Dubey (2010) in order to isolate the *T. gondii* infective stages.

Maintenance of *T. gondii* strains: The RH and equine *T. gondii* isolate was maintained in the laboratory by serial passage in mice according to the procedures of Johnson *et al.* (1979).

Polymerase Chain Reaction (PCR)

DNA extraction: Genomic DNA was extracted from *T. gondii* RH strain (positive control) using a commercially available kit (Dneasy blood and Tissue kit, Qiagen Co., Cat. No. 69504) with the following modifications to the manufacturer's protocols: (1) Tachyzoites was first suspended in 300 µL of ATL buffer (included in the same kit). (2) Lysed tachyzoites suspensions were then incubated with 20 µL proteinase K for 3 h at 56°C, followed by incubation with 300 µL ATL buffer at 70°C for 10 min with vortexing for 10 s every 3 min. DNA was purified through the columns according to the manufacturer's protocol and eluted in 50 µL of the supplied AE buffer and then stored at -20°C. Concerning blood samples, it was collected in test tubes containing heparin as an anticoagulant then digested using the same kit using the blood protocol recommended by the manufacturer prior to assay by PCR.

PCR amplification: Primers used for PCR were targeting the repetitive 35-fold B1 gene (Burg *et al.*, 1989). The outer primers were only used in this study. The reactions were set up to a final volume of 25 µL containing 2 µL of DNA sample (standard controls) or 5 µL of blood specimen DNA, 1 µL of each primer (100 pmol) and 12.5 µL Pyro-Start™ Fast PCR Master Mix (Fermentas Co., Cat. No. Ko211). PCR was performed in a PTC-100 Peltier Thermal Cycler (MJ Research, Incline Village, USA). The first cycle included 1 min of denaturation at 95°C. This first step was followed by 39 cycles of 2s of denaturation at 94°C, 5s of annealing at 48°C and 25s of primer extension at 72°C and a final extension step of 72°C for 10 min. A 10 µL aliquot of the amplified product was analyzed on 1% agarose gel and stained with Ethidium bromide. Every PCR run included positive and negative controls.

Serological tests used for detection of *T. gondii* antibodies: The collected sera were examined serologically for detection of *T. gondii* antibodies using the following 3 serological tests:

- **Latex Agglutination Test (LAT):** The antigen prepared through sensitization of latex particles with *T. gondii* sonicated tachyzoites as procedures of Lunde and Jacobs (1967). The latex agglutination test was adopted as methods of Holliman *et al.* (1989), using negative and positive reference serum samples and reactions were performed by adding a drop of latex solution into a drop of serum on a microscope glass slide and mixed by side movement of slide. The agglutination appears within 2-3 min in positive cases
- **Enzyme Linked Immunosorbent Assay (ELISA):** The tachyzoite antigen prepared as procedure described by Waltman *et al.* (1984), briefly, tachyzoites were repeatedly freeze and thawed, sonicated and centrifuged at 12,000 rpm for 45 min at 4°C. The supernatants were separately collected and its protein content was determined by the method of Lowry *et al.* (1951). The optimum antigen, serum and conjugate concentrations were determined by checkerboard titration and test procedures carried out according to the method described by Lind *et al.* (1997)
- **Modified Agglutination Test (MAT):** The formalized killed whole tachyzoites of *T. gondii* (equine origin) was prepared according to the method described by Desmonts and Remington (1980). The procedures were carried out according to the method described by Dubey and Desmonts (1987) at a dilution of 1: 25

Statistical analysis: The data of the various serological tests were statistically analyzed. The sensitivity and specificity of each test were determined by comparing its results with that of PCR as a reference test according to the method described by Waltman *et al.* (1984) and Dubey *et al.* (1995).

RESULTS

Prevalence of *T. gondii* infection in sport horses using PCR and serologic assays: Examination of the 240 serum samples of sport horses by PCR, LAT, MAT and ELISA revealed that 129 (53.8%), 125 (52.1%), 122 (50.8%) and 94 (39.2%) were positive reactors with *T. gondii* respectively, which at the same time was considered the percentage of infection (Table 1, Fig. 1).

Prevalence of *T. gondii* in relation to breed, sex and reproductive condition: Imported breed horses showed high prevalence (73.1%) followed by (58.5%) in native breeds while Arabian breeds revealed lowest prevalence (44.4%). The prevalence was higher (60.8%) in female represented by 10.8, 12.4 and 29.9% in fillies, pregnant and repeat-breeders, respectively. On other hand, lower prevalence in males (23.9%) represented by 0, 8.7 and 15.2, in colts, stallions and racers, respectively (Fig. 2).

Comparison between the PCR and serological tests: The sensitivity and specificity of LAT, MAT and ELISA serological tests were calculated by comparing their results with those of PCR test and revealed that MAT had the highest sensitivity (93.8%) followed by LAT (91.5%) and the lowest

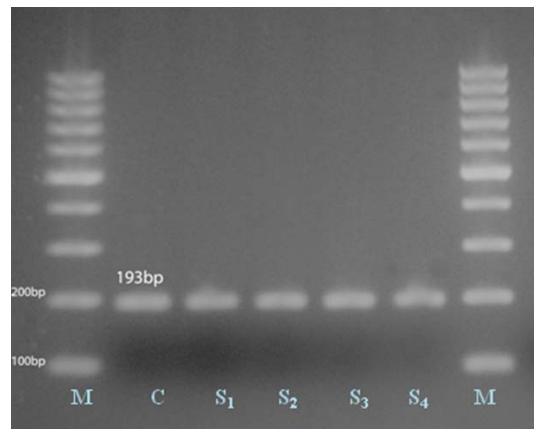


Fig. 1: Detection of *T. gondii* in horses blood samples by PCR. M: Mol. wt. marker (100 bp ladder); C: Positive control; S₁-S₄: Positive PCR *T. gondii* samples at 193 bp

Table 1: Prevalence of *T. gondii* infection among sport horses using PCR and different serological tests

Test	Positive reactors		Negative reactors	
	No.	%	No.	%
PCR	129	53.8	111	46.2
LAT	125	52.1	115	47.9
MAT	122	50.8	118	49.2
ELISA	94	39.2	146	60.8

*No. of samples examined by each test were 240

Table 2: Comparison between the PCR and serological tests used for detection of *T. gondii* infection

Test	Positive by both PCR and compared serological test	Negative by both PCR and compared serological test	PCR		Sensitivity (%)	Specificity (%)
			Positive	Negative		
LAT	118	98	129	111	91.5	88.3
MAT	121	102	129	111	93.8	91.9
ELISA	92	103	129	111	71.3	92.8

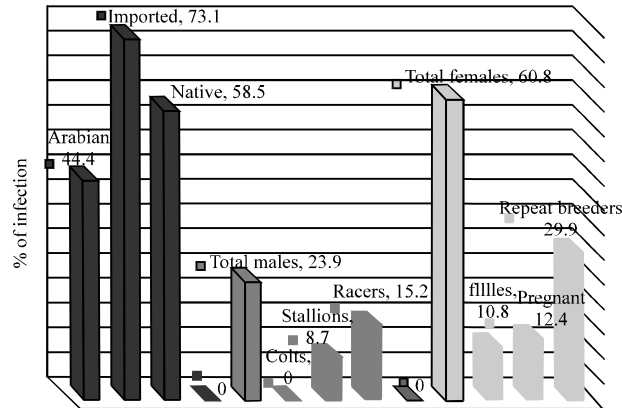


Fig. 2: Prevalence of *T. gondii* infection in relation to breed, Sex and Reproductive condition

sensitivity by ELISA (71.3%). On the other hand ELISA had the highest specificity (92.8%) followed by MAT (91.9%) and the lowest specificity was by LAT (88.3%) (Table 2).

DISCUSSION

In the present work, Prevalence of *T. gondii* infection in sport horses was higher by PCR (53.8%) followed by LAT (52.1%), MAT (50.8%) and lowest prevalence by ELISA (39.2%), nearly similar results of incidence of toxoplasmosis in equine (48.1, 43.7 and 34%) were recorded by Ghazy *et al.* (2007) in farmed horses in Egypt, Alanazi and Alyousif (2011) in horses for sporting purposes in the Province of Riyadh, Saudi Arabia and Dangoudoubiyam *et al.* (2011) in Costa Rica, respectively whereas lower incidence rates (1, 6.9 and 23%) was obtained by Ugula *et al.* (1990) in Swedish horses, Dubey *et al.* (1999) in North America and Bartova *et al.* (2010) in Czech Republic, respectively. However higher incidence rates (97.1, 65.6 and 71.2%) were recorded by Mayer *et al.* (1987) in Argentina, El-Ghaysh (1998) in Egypt and Hajialilo *et al.* (2010) in Iran, respectively.

The difference between the obtained results of PCR and serological tests during the present study and those reported by other investigators might be attributed to the host-parasite relationship which depends upon the virulence of *T. gondii* strain, time of exposure to infection, biology of the parasite and the immune status and age of the infected horses in different localities. This is agreed with that concluded by Tassi (2007) who added the prevalence of equine *Toxoplasma* infections under natural conditions may vary from 0 up to 90% depending on the sensitivity of the test used, age of the animals, geographic area, hygienic conditions of the farms and farm management.

Prevalence of *T. gondii* infection in relation to breed in this study indicated that the prevalence in Arabian breeds was lower (44.4%) than (58.5%) in native and (73.1%) in imported breeds. This is in agreement with results previously obtained by Riemann *et al.* (1975) who found that the

prevalence of *T. gondii* was lower in Arabian (19%) than in Paint (22%) and Thoroughbred (24%) horses in USA. Also, Aganga *et al.* (1983) recorded that local breeds of horses in Nigeria showed lower incidence of *T. gondii* (33.0%) than by imported breeds (38.5%).

However in the present work, high prevalence of *T. gondii* infection was recorded in females (60.8%) represented by 10.8, 12.4 and 29.9% in fillies, pregnant and repeat-breeders, respectively. Similar very little studies have shown sex and reproductive conditions associated with toxoplasmosis in horses. Cusick *et al.* (1974) reported high prevalence level of toxoplasmosis in horses have a history of abortion, Roberto *et al.* (1983) isolated *T. gondii* from liver of an aborted foal and Turner and Savva (1990) recorded *T. gondii* DNA in lesions from placenta of a mare.

Little is known about the specificity and sensitivity of serological diagnosis of *T. gondii* infection in equine. Highest sensitivity (93.8%) by MAT, highest specificity by ELISA (92.8%) and lowest specificity (88.3%) by LAT were obtained in this study when the data of the serological tests were compared with that of the PCR, as a reference test for toxoplasmosis. Also Dubey (2010) concluded that detection of *Toxoplasma* DNA by PCR was considered the most specific and standard test by which all other tests should be judged. LAT revealed lowest specificity, is frequently produce false positive LAT titers and so technical considerations involving accuracy of reading weakly positive reactions could account for some of the differences (Holliman *et al.*, 1989). On other hand, ELISA demonstrates great specificity, is quantitative, low cost and may be automatically adopted, but requires further refinement with regard to procedures and standardization of the antigen used (Dubey *et al.*, 1995). Regarding MAT, Dubey (1997) and Shaapan *et al.* (2008) found that MAT has the highest sensitivity among all serological tests for detection of *Toxoplasma gondii* infection in naturally infected sows and sheep, respectively which is easy to perform and does not require sophisticated equipment.

CONCLUSION

It is of interest to point out that, the present report is the first on detection of *T. gondii* infection in sport horses in Egypt. The high prevalence of this zoonotic parasite suggests that the risk of humans contracting infection from horses seems extremely high, although consumption of horse meat is uncommon among Egyptians. However, participation of horses in the transmission cycles is possible as a source of infection either for vectors or definitive hosts. In addition this study suggested that MAT alone or with LAT can be used as a highly sensitive screening test followed by PCR as a specific confirmatory test for diagnosis of toxoplasmosis in Equines.

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