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Research Article
Molecular Detection and Genotyping of *Toxoplasma gondii* from Egyptian Isolates

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Abstract

Background and Objective: *Toxoplasma gondii* is a zoonotic opportunistic cyst-forming protozoan has usual clonal population of three virulent human and animal genotypes which differ according to their virulence in mice and epidemiological patterns. Animals are of zoonotic impact and regarded as high prevalent sources for human toxoplasmosis via their meat containing viable *T. gondii* tissue cysts. However, serological assays are not enough for approving virulent *T. gondii* tissue cysts or confirm virulent types harboring animals so the current study was aimed to evaluate the zoonotic hazard of *T. gondii* seropositive Egyptian animals and aborted women via mice viability test and genotyping as excellent bio-indicators for virulent strains. **Methodology:** Both blood and the matching tissue samples were collected from 76 aborted women and 755 farm animals (254 sheep, 293 goats, 45 cows, 41 buffalos, 34 camels and 88 free range poultry). All samples were assayed serologically by using ELISA. The tissue samples which were identical to seropositive sera were digested and microscopically examined besides exposed to DNA detection and genotyping. The microscopic confirmed bradyzoites containing ones were bio-assayed through intra-peritoneal passage in mice as viability test. **Results:** The total seropositive percentages and the suspected microscopic examined samples plus to DNA percentages detected and succeeded isolates via mice viability test were (6, 8, 4, 0, 0, 2 and 2) corresponding to (women, sheep, goat, cows, buffaloes, camels and free range poultry) respectively. Genotyping confirmed 22 isolates (2 type- I, 13 type- II and 7 type- III). **Conclusion:** The mice viability and DNA approve along with genotyping were signify *T. gondii* virulent types 1-3 viable cysts from Egyptian women, animals and poultry and reflect zoonotic bio-hazard of public health concern.

Key words: *T. gondii*, aborted women, farm animals, poultry, ELISA, genotyping, mice viability test, zoonotic bio-hazard

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular tissue cyst-forming protozoan, capable of infecting and multiplying in all warm-blooded animals, birds and humans with worldwide distribution[1]. It exists in three infective stages, the acute tachyzoite stage which is responsible for the materno-fetal pass, the dormant life-persistent tissue cyst containing bradyzoites which forms particularly in neural and muscular tissue including uterus and placenta, while the environment oocysts are developed only in the feline's gut and disseminated in their feces[2]. Human toxoplasmosis is a typical meat borne zoonosis[1], postnatal humans infection is mostly via eating insufficient cooked meat harboring tissue cysts, which could persist viable in animal tissues[3]. Also, persons or animals can harbor bradyzoites through food or water contaminated with environment oocysts shed in cat feces.

Toxoplasmosis exceeds opportunistic human impact sequence to cerebral[4], congenital[5] and ocular toxoplasmosis[6,7]. Also, maternal-fetal pass in pregnant women possible develop abortion, stillbirths, mental retardation, plus neurological sequel of congenital malformations and ocular disorders[8], this usually set consequence to estrogen/progesterone shift[9] or related to impermanent gravidity hyperglycemia[10]. Also, may be potential to anti-inflammatory or corticosteroids therapy[11]. The disease ranks among the 10 most opportunistic pathogens and the forth deadly pathogen in immune-suppressed patients[2].

The animals and humans isolates are types, I, II and III which differ according to their virulence in mice and epidemiological patterns[12]. Type I strain is highly virulent in mice and mainly recorded in patients with ocular toxoplasmosis and more likely to cause severe human toxoplasmosis[13,14]. Type II strain is of moderate virulence for mice but the most common strain associated with human infections which creates chronic persistent tissue cysts[15]. Moreover, latent cystogenic (cyst-forming) types I and II strains have been recorded in patients with congenital disease and AIDS patients[16,17]. Type III has low-virulence for mice and is uncommon human type but the most frequent strain from animals[18].

A variety of different methods has been successively used for genotyping of *T. gondii* isolates with distinct advantages and disadvantages. Early studies of strain typing were based on multilocus enzyme electrophoresis[19] and later focused on microsatellite markers[14]. Also, randomly amplified polymorphic DNA and Restriction Fragment Length Polymorphism (RFLP) analysis of specific genetic loci has been widely used for *T. gondii* genotyping[12].

Unfortunately, inadequate genotyping data of *T. gondii* isolates in Africa and middle East including Egypt is due to difficulty in obtaining viable isolates because of poor laboratory facilities in most African countries and the information is mostly based on a few PCR-RFLP markers[20]. Furthermore, there have been insufficient researches on Egyptian meat producing animals and poultry concerning toxoplasmosis[21,22]. Serological assays aren't enough for confirming zoonotic biohazards of *T. gondii* virulent types in animals. Therefore, the present study aimed to evaluate the zoonotic bio-hazard of *T. gondii* seropositive Egyptian animals and aborted women via mice viability test and genotyping, as excellent bio-indicators for virulent strains having zoonotic impact.

MATERIALS AND METHODS

Blood and tissue samples: Both blood and the matching tissue samples were collected from 76 aborted women, 254 sheep, 293 goats, 45 cows, 41 buffaloes, 34 camels and 88 free range poultry respectively from Cairo, Giza and Qaliubiya governorates (Egypt).

Serological assay: Sera were separated, labeled and kept at -20°C until examined serologically against toxoplasma infection by ELISA which was carried out according to the method described by Aubert *et al.*[23] using soluble crude antigen prepared from *Toxoplasma* RH strain tachyzoites.

Digestion of meat samples: Tissue samples were prepared as described by Shaapan and Elfadaly[24], 20 g of the equivalent tissue samples from diaphragm and thigh muscles collected from animals during slaughtering in abattoirs and collected from various batches of free range poultries from diverse villages in rural areas in Egypt. The tissues were cut into small cubes about 5×5×5 cm and classified into two groups, the 1st was frozen at -80°C for further DNA extraction while the 2nd group was exposed to pepsin digestion and then bio-assayed for both microscopic examination and mice viability test.

Mice viability test: The test procedures were done according to Elfadaly *et al.*[9], a total number of 48 seronegative Swiss Webster Albino mice obtained from Laboratory Animal House, National Research Centre, Egypt. The inoculated mice were followed up daily for any apparent clinical signs of febrile response or acute toxoplasmosis with the exclusion of dead

mice before 48 h of inoculation. The predicted signs may be varied between ascites, roughcast hairs, tottering gait, hunched appearance, with evidence of early emaciation and dehydration or death usually within 72-84 h (with highly virulent types) or nervous manifestations and partial off food (with moderately virulent types). Depending on the virulence of the isolate, the procedure will be continued. If ascites occurred, peritoneal exudates were collected from ascetic mice within 72-84 h DPI and microscopic examination for tachyzoites was done. If mice didn't show ascites they were sacrificed within 15th day post-inoculation by cervical dislocation, according to Ajzenberg *et al.*[14].

Detection of *T. gondii* by PCR: Part of frozen tissue samples at -80°C without preservatives will be processed for molecular studies and genotyping. Genomic DNA of *T. gondii* from the collected tissue samples was extracted by a Kit (ViVantis Co., Malaysia) according to the manufacturer instructions. The B1 gene was amplified[25], using primers 1 (5′-TCG GAG AGA GAA GTT CGTCGC AT-3′) and 2 (5′-AGC CTC TCT CTT CAA GCA GCG TA-3′). The following reaction mixture was added in a 0.2 mL PCR tubes: DNA template (100 ng μL^{-1}), 10 μL; Taq polymerase (5 u μL^{-1}) 1 μL; 10x enzyme buffer, 2 μL; dNTPs, 0.8 μL; each primer, 1 μL and Bidest. water to 20 μL. The mixture was briefly spine and placed in the thermal cycler (T gradient, Biometra, Germany), which was programmed as follow: Initial denaturing (95°C/2 min) and 40 cycles consisting of denaturing (95°C/1 min), annealing (55°C/30 sec), extension (72°C/45 sec) and final extension (72°C/10 min). The PCR product was electrophoresed at 80 v/15 min and finally examined using UV trans-illuminator. About 100 bp DNA ladder (Finnzymes) was used as a marker. Positive control was DNA from RH strain and negative control was distilled water in presence of primers.

Genetic characterization: *Toxoplasma gondii* DNA was extracted from tissues as described by Lehmann *et al.*[26]. The PCR-RFLP genotypes of the SAG2 locus were used to determine the genetic type. Samples were analyzed at the SAG2 locus by using a nested PCR approach that separately amplified the 5' and 3' ends of the locus. The 5' end of the locus was amplified by standard PCR for 40 cycles with the primers SAG2.F4 (5'-GCTACCTCGAACAGGAACAC-3') and SAG2.R4 (5'-GCATCAACAGTCTTCGTTGC-3') at an annealing temperature of 65°C. The resulting amplification products were diluted 1/10 in water and a second amplification of 40 cycles was performed with the internal primers SAG2.F (5'-GAAATGTTTCAGGTTGCTGC-3') and SAG2.R2

(5'-GCAAGAGCGAACTTGAACAC-3') by using 1 mL of the diluted product as the template. The amplified fragments were purified with Quia Clean (Qiagen Inc.) and digested with Sau3AI. The 3' end of the locus was similarly analyzed with the primers SAG2.F3 (5'-TCTGTTCTCCGAAGTGACTCC-3') and SAG2.R3 (5'-TCAAAGCGTGCATTATCGC-3') for the initial amplifications and the internal primers SAG2.F2 (5'-ATTCTCATGCCTCCGCTTC-3') and SAG2.R (5'-AACGTTTCACGAAGGCACAC-3') for the second round of amplification at an annealing temperature of 63°C. The resulting amplification products were purified with Quia Clean and digested with *Hha*I. The PCR products and the restriction fragments were analyzed by 2% agarose gel electrophoresis. Restriction digestion of 5'-end-amplified products with Sau3AI distinguished the type III strain from types I and II strains and *Hha*I digestion of the 3'-end-amplified fragments differentiated types I and III strains from type II strains. In order to avoid possible contamination, several measures, such as separate space to set up PCRs, filter tips, etc., were taken, as well a negative control (no DNA) and positive controls from different strains of *T. gondii* were used in order to locate any possible contamination.

Ethical approval: The study was approved ethically by the Medical Research Ethics Committee-National Research Centre, Egypt under registration number 1-2/0-2-1.2012.

RESULTS

Table 1 shows that total seropositive percentages were (42.1, 64.2, 43.3, 35.5, 17.1, 26.5 and 37.5), total suspected microscopic examinations were (42.3, 46, 38.6, 12.5, 0, 44.4 and 27.3), DNA detection percentages (32.7, 52.7, 41.7, 18.8, 0, 55.5 and 36.4) and total succeeded isolates via mice viability test (6, 8, 4, 0, 0, 2 and 2) corresponding to (women, sheep, goat, cows, buffaloes, camels and free range poultry) respectively.

Genotyping: The target regions for amplification were shortened to encompass only the relevant polymorphisms. Primers were selected to separately amplify the 5' and 3' ends of the *T. gondii* SAG2 locus as 241 and 221 bp products, respectively. Digestion of the 5' amplification products with Sau3AI distinguished allele 3 (type III strains) from alleles 1 and 2 (type I and II strains) and digestion of the 3' amplification products with *Hha*I distinguished allele 2 (type II strains) from alleles 1 and 3 (type I and III strains). In this
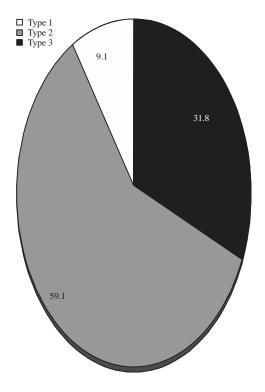
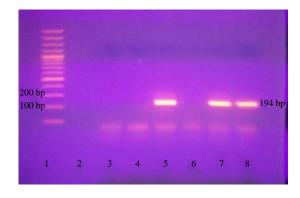Fig. 1: Percentages of genotyped positive samples

Fig. 2: Detection of *Toxoplasma* genome by PCR using *Toxoplasma* gene B1 primers

Lane 1: 100 bp DNA ladder, lanes 7 and 8: Positive isolates (194 bp), lane 5: Reference RH strain, lanes 2, 3, 4 and 6: Negative specimens

study, samples were typed as 2 (9.1%) type I, 13 (59.1%) type II and 7 (31.8%) type III using PCR-RFLP analysis, the genotypes (Table 1, Fig. 1-4).

Significance and interpretation of the results: This study point out to the zoonotic hazard of *T. gondii* seropositive Egyptian animals and aborted women via mice viability test and genotyping, as excellent bio-indicators for virulent strains.

Table 1: Results of ELISA, microscopic examination, DNA detection, mice viability test and genotyping positive samples

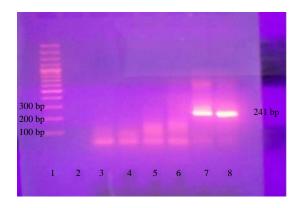| Groups	No. of blood and tissue samples	Sero-positive/ positive (%)	Microscopic exam/ Sero-positive (%)	DNA detection/ Seropositive (%)	Mice viability test/ microscopic exam (%)	Genotyping I	II	III
Sheep	254	163/254 (64.2)	75/163 (46)	86/163 (52.7)	8/75 (10.7)	0	6	2
Goat	293	127/293 (43.3)	49/127 (38.6)	53/127 (41.7)	4/49 (8.2)	0	0	4
Cows	45	16/45(35.5)	2/16 (12.5)	3/16 (18.8)	0/16	0	0	0
Buffalos	41	7/41 (17.1)	0/7 (0)	0/7 (0)	0	0	0	0
Camels	34	9/34 (26.5)	4/9 (44.4)	5/9 (55.5)	2/4 (50)	0	1	1
FR poultries	88	33/88 (37.5)	9/33 (27.3)	12/33 (36.4)	2/9 (22.2)	0	2	0
Total for animals	755	355/755 (44.34)	139/355 (39.1)	159/355 (44.8)	16/139 (11.5)	0	9	7
						0	9/16 (56.2)	7/16 (43.8)
Women	76	32/76 (42.1)	22/52 (42.3)	17/52 (32.7)	6/22 (27.3)	2	4	0
						2/6 (33.3)	4/6 (66.7)	0

Fig. 3: Amplicon of the 5' of the *T. gondii* SAG2 locus as 241 bp product
    Lane 1: 100 bp DNA ladder (Fermentas), lanes 7 and 8: Positive isolates (~241 bp), lane 2-6: Negative specimens
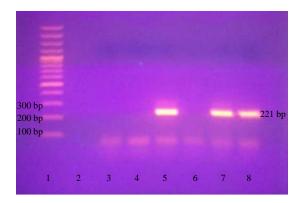

Fig. 4: Amplicon of the 3' end of the *T. gondii* SAG2 locus as 221 bp product
    Lane 1: 100 bp DNA ladder (Fermentas), lanes 5, 7 and 8: Positive isolates (~221 bp), lanes 2-4 and 6: Negative specimens

Bio-assay through intra-peritoneal passage in mice as viability test suspected for *T. gondii* isolates and DNA percentages were detected. The obtained results concluded that mice viability and DNA approve along with genotyping were signify *T. gondii* virulent types I, II and III viable cysts from Egyptian women, animals and poultry and reflect zoonotic bio-hazard of public health concern.

DISCUSSION

High prevalence (about 70%) of Egyptians is *T. gondii* seropositive, perhaps connected to consuming undercooked meat or poultry rather than oocysts contaminated food or water[10]. So, recent etiological investigation concerning risk factors plus genotyping of *T. gondii* local Egyptian isolates

should be updated to explain the illogic data of high human sero-prevalence. In the present study, B1 gene choice as target to PCR amplification was established on the explanations made by other studies[27].

Isolates of *T. gondii* type-I are poor forming either meat tissue cyst or environmental oocysts (cat feces) and mainly transmitted via placental diffusion, it is highly lethal to mice with high zoonotic concern and likely to cause severe congenital and ocular human toxoplasmosis[13]. In contrast, latent types II and III are highly cystogenic (cyst-forming) and well shed oocysts. Thus, they essentially spread through meat cysts or sporulated oocysts. Type III is animal strains, rarely seen in human toxoplasmosis[28]. In the present study, genotyping of 6 *T. gondii* women isolates confirmed two of type-I (33.3%) and four of type-II (66.7%). Where, 2 isolates of type-I was recorded only in women, denote great prospect placental transmission. A study in 2008 at Ain Shams University Hospitals on 38 female patients with abortion and intrauterine fetal death, genotyping proved 38 isolates, type II was the most prevalent genotype found in 33 (87%). Type I was found in 5 (13%) of the isolates, whereas genotype III was not never found[29].

In the existing study, higher possibility of zoonotic transmission was established. Where, genotype-II which is the main women isolates has been detected in meat of Egyptian producing animals (6/8 in sheep, 1/2 in camels and 2/2 in poultry). Thus, mutton in the current study represents considerable zoonotic risk. While free range poultry indicated the probable higher zoonotic risk through 2 isolates of genotype-II. The results of this study agree with Dubey *et al.*[21] who detected high prevalence in Egyptian free range chickens (40.4%) and ducks 15% from rural area surrounding Giza. This probably due to free range poultry is mainly infected via environmental oocysts. Additionally, they like omnivore's classes, possible engulf raw meat residues[30]. Also, our results are accepted through other studies which validated the vast majority of virulent Egyptian sheep and poultry isolates are corresponding to type II strain which is the predominant human types[31]. Moreover, from São Paulo state, Brazil, *T. gondii* was isolated from tissue homogenates of 16 sheep, where 6 of the 16 isolates killed 100% of infected mice[32]. In contrast, we could not isolate any of *T. gondii* tissue cyst from the sero-positive cattle (cows and buffaloes), perhaps due to low susceptibility and higher resistance of those species or owing to incompatibility among the taken tissue samples and the parasite harboring ones.

In the present study, mice viability test of meat producing animal identify potential zoonotic hazard via three virulent strains, I, II and III. But, more one type may be harboring the same human or animal hosts[33]. However, the higher virulent types usually hidden the biological properties of less virulent ones. Thus, the practice of parasite isolation via mice viability possible results a single strain even several strains were found in the inoculum. So, genotyping is more accurate for strain recognition but mice viability test is essential for morbidity and virulence discovery.

Classically, consumption of undercooked meat has been documented as the major risk factor, followed by dusting oocysts and placental diffusion[2]. Therefore, many reports have indicated that human toxoplasmosis mainly took place in many localities free from cats[34]. This is in contrast to farm animals that are usually infected by cohabitation with cats[31]. Where, intensifying awareness against undercooked meat minimizing human prevalence world-wide. So, the incidence of human toxoplasmosis completely differs from country to other according to the socio-economic pattern of meat feeding behavior. Wherever, various communities cover diverse culture habits are contributed to the zoonotic aspects for spreading toxoplasmosis[35].

Insufficient cooked meat is the main risk factor but not the absolute one. Occasionally some strains may survive deep-freezing[36]. Microwaving or superficial heating does not destroy all bradyzoites and remain viable in refrigerated carcasses (1-4°C) or minced meat for up to 3 weeks[37], also survive freezing at temperatures between -1 and -8°C for longer than a week and cyst containing bradyzoites remain viable and infective for as long as the meat is edible[38], Accordingly, changing our eating behavior possible minimizes the high incidence of human toxoplasmosis. Where, dormant cysts in meat was rendered non-viable when internal temperatures had reached 67 or -12°C freeze for 1 day[35].

CONCLUSION

Bio-viability and molecular recognition along with genotyping of *T. gondii* Egyptian isolates from human and animal hosts are on target bio-indicators, signify zoonotic impact through virulent strains and must be focused for avoiding feeding on under cooked meat of animals or birds.

SIGNIFICANCE STATEMENTS

This study evaluate the zoonotic hazard of *T. gondii* seropositive Egyptian animals and aborted women via mice viability test and genotyping, as excellent bio-indicators for virulent strains. However, the microscopic confirmed bradyzoites containing ones were bio-assayed through intra-peritoneal passage in mice as viability test. The total seropositive percentages and the suspected microscopic examined samples plus to DNA percentages were detected. Also, the total succeeded isolates via mice viability test were (6, 8, 4, 0, 0, 2 and 2) corresponding to (women, sheep, goat, cows, buffaloes, camels and free range poultry) respectively. Genotyping confirmed 22 isolates (2 type-I, 13 type-II and 7 type-III). Mice viability and DNA approve along with genotyping were signify *T. gondii* virulent types I, II and III viable cysts from Egyptian women, animals and poultry and reflect zoonotic bio-hazard of public health concern.

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