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## Biological Assay of *Toxoplasma gondii* Egyptian Mutton Isolates

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### ABSTRACT

Mutton signifies one of the most prevalent sources for human toxoplasmosis. However, sheep serological assays don't categorize the virulent strains initiating antibodies, so the biological bioassay of Egyptian mutton isolates with reference to their pathogenicity in both mice and kittens were done in this study for indicating to how extent their zoonotic bio-hazard. A total number of 280 of each sheep blood and tissue samples were collected during slaughtering at Cairo abattoir, Egypt. Sera assayed using Latex Agglutination Test (LAT) and immunosorbant assay (ELISA) and their corresponding mutton samples were microscopically examined after pepsin digestion for detection of *Toxoplasma gondii* infection. The sero-positive percent of the naturally infected sheep was 50.4 and 61.4 by LAT and ELISA, respectively, 47.9% of samples were confirmedly positive in both LAT and ELISA results. The microscopical examination revealed that only 28 out of 134 (20.9%) of the confirmed sero-positive animals by both tests were found harboring *T. gondii* tissue cysts in their mutton samples, while high percentage of confirmed sero-positive animals (79.1%) (106 out of 134) were biologically tissue cysts free mutton. Biological typing of the 28 *T. gondii* sheep isolates with reference to mice and kittens' bioassay indicated that 10.7, 50, 21.4 and 17.9% were type I, II, III and avirulent strains, respectively. The high *T. gondii* infection rate resulted in this study concludes that the feeding of under cooked mutton is a bad health habit as a source for human toxoplasmosis moreover; the *T. gondii* virulent strains obtained by mutton bioassay indicated that not all sero-positive sheep are connecting zoonotic bio-hazard through their mutton strains.

**Key words:** *Toxoplasma gondii*, Egyptian mutton isolates, LAT, ELISA, bioassay in mice and kittens

### INTRODUCTION

*Toxoplasma gondii* is an obligate intracellular tissue cyst-forming coccidian protozoan that has potential zoonotic impact and ranks among the most commonly occurring opportunistic pathogens (Nissapatorn *et al.*, 2007). Only feline species are the final host shed millions of the environmental resistant oocysts, leading to an oral infection particularly for herbivorous animals with subsequent harboring tissue cysts in their meat (Weiss and Dubey, 2009). Concerning humans, the protozoon is vertically transmitted maternofetal via acute stage tachyzoites that may also diffuse via blood transfusion, tissue transplants or unpasteurized milk. While horizontal transmission may involve ingesting either the environmentally sporulated oocysts or the dormant chronic tissue cysts that are inhabitant in tissues of food animals (Fromont *et al.*, 2009).

The high Egyptian sero-prevalence of *T. gondii* during the last years (40-60%) could be linked to the primary infection through undercooked or processed meat rather than ingestion of food or water contaminated with oocysts shed via cats (Elfadaly, 2007; Sabry and Reda, 2008). Supported concept, that human infection took place in many localities free from cats and feeding habits among consumers have been extra changed during the last years, due to the establishment of restaurants serving quick meat meals that may be insufficiently cooked (Cook *et al.*, 2000).

Meat tissue cysts, especially harboring mutton represent the main source for human toxoplasmosis (Bonyadian *et al.*, 2007); in a study of 131 mothers in the USA who had given birth to children infected with *T. gondii*, 50% recalled have eaten uncooked meat (Boyer *et al.*, 2005). The dormant tissue stage bradyzoites have been demonstrated more prevalent in mutton, beef and chicken meat than in goat and buffaloes meat (Aspinall *et al.*, 2002; Dubey *et al.*, 2002).

The vast majority of animals and humans isolates are still now belonging to only 3 clonal lineages designated as types I, II and III. Human isolates are mainly from type II and are most common in chronic infected animals used for human consumption (Howe *et al.*, 1997). Fifteen genotypes among 57 *T. gondii* isolates were identified in lambs from Maryland and the phylogenetic analysis indicated that the clonal type II lineage and its closely related genotypes accounted for 68% (39 of 57) of the isolates, while the type III lineage accounted for 14% (8 of 57) of the strains and was the second most prevalent genotype (Dubey *et al.*, 2008). Although type I strains are relatively rare in animals but may be more likely to cause severe human congenital toxoplasmosis and ocular disease (Grigg and Boothroyd, 2001).

Extraordinary tools were used to analyze the biological diversity and typescript of animal isolates with reference to their pathogenicity to mice, diverse geographic isolates originating from different parts of the world are necessary to appreciate the real strain varieties (Ajzenberg *et al.*, 2002). Tissue bioassay of 82 seropositive sheep sera from Brazil, using mice revealed isolation of *T. gondii* from only, 16 (19.5%). Six of the 16 *T. gondii* isolates killed 100% of infected mice, so these results indicate that asymptomatic sheep can harbor mouse-virulent *T. gondii*; hence, they can serve as a source of infection for humans (Dubey, 2009).

However, many isolates originating from remote areas of South America were analyzed, little is known of the biological characters of isolates from the Middle East and Africa (Dubey, 2009) and moreover, there have been relatively few recent reports on Egyptian small ruminants concerning toxoplasmosis (Shaapan *et al.*, 2008; Barakat *et al.*, 2009; Shaapan *et al.*, 2010). Therefore, the present study aimed to characterize the biological typescript of virulent Egyptian mutton isolates with reference to their pathogenicity in both mice and kittens, indicating to how extent the zoonotic bio-hazard is due to mutton harboring virulent types.

## **MATERIALS AND METHODS**

**Sheep blood and tissue samples:** This study was conducted from 1-2011 to 4-2011. A total of 280 blood and corresponding tissue samples from the diaphragm were collected from sheep during slaughtering at Cairo abattoir, Egypt. Sera were separated, labeled and kept at -20°C until serologically assayed.

***Toxoplasma gondii* strains:** Three strains; RH, ME-49 and Prugniaud (PRU) *T. gondii* strains secured in the Zoonotic Diseases Department, National Research Center, Egypt, were used as type's control mutton isolates, representing types I, II and III.

### Serological assays

**Latex agglutination test (LAT):** The collected sheep sera were tested for *Toxoplasma* infection by the LAT according to the manufacturer's instructions (Toxocheck-MT; Eiken Chemical, Tokyo, Japan). It is considered positive when agglutination observed at dilutions of 1:64 and greater.

**Enzyme Linked Immunosorbent Assay (ELISA):** *Toxoplasma gondii* RH strain Tachyzoites soluble crude antigen was prepared as described by Abdollahi *et al.* (2009). The original ELISA procedures were carried out according to the method described by Aubert *et al.* (2000).

**Digestion and microscopical examination of mutton samples:** The 20 g of diaphragmatic muscle samples from each of the 280 sero-examined sheep were separately digested by pepsin according to method of Sharma and Dubey (1981) followed by microscopically examined at low and high powers for the presence of *T. gondii* tissue cysts containing the bradyzoites.

### Biological typing of mutton isolates from the sero-positive animals

**Bioassay in mice:** Each sample of microscopically confirmed tissue cysts was intra-peritoneal inoculated into 2 mice. The animals were inspected daily for ascites, followed by signs of tottering gait, hunched appearance; evidence of early emaciation and a sample of peritoneal exudates was removed and inspected for tachyzoites by microscopic examination (Buxton *et al.*, 1988).

**Bioassay in kittens:** Fifteen sero-negative kittens were used; 12 kittens 3 ones each were orally inoculated with tissues of mice confirmed to harbor tachyzoites or bradyzoites corresponding to the virulent types I, II, III and the avirulent ones, while 3 remaining kittens still un-inoculated control ones. Feces examined daily for oocysts determination. The oocyst characters concerning their numbers, shedding times and courses were detected for each isolate according methods described by Dubey and Beattie (1988).

**Data monitoring:** The biological typing of mutton isolates was done according to Howe *et al.* (1997) who classified the vast majority of isolates studied until now to belong to only 3 clonal lineages designated types I, II and III based on LD, ability of tissue cyst formation and oocysts characters.

## RESULTS

The sheep sera showed sero-positive results with percentage of infection with *Toxoplasma* 50.4% (141/280), 61.4% (172/280) and 47.9% (134/280) subsequent to LAT, ELISA and by both LAT&ELISA, respectively. The digested 134 mutton samples corresponding to the confirmed both LAT and ELISA sero-positive animals showed that 28 (20.9%) of them were contained tissue cysts by microscopic examination (Table 1).

All (28) mutton samples containing tissue cysts were biologically assayed in both sero-negative mice and kittens compared with the control types; 3 (10.7%), 14 (50%), 6 (21.4%) and 5 (17.9%) were corresponding to type I, II, III and avirulent one, respectively (Table 2).

*T. gondii* isolates biologically classified according to bioassay in mice and kittens into the following strains: Type I (RH) strain had highly virulent character, where 100% deaths [LD<sub>100-1</sub>] of injected mice within 3-5 DPI, tissue cysts had no time to be formed and no or few shed of atypical *T. gondii* oocysts, only scattered free sporozoites like stage were detected in cats' feces for few days

Table 1: Prevalence of *T. gondii* infection among sheep sera and presence of tissue cysts in confirmed +ve serologically corresponding mutton samples

Test	No. examined	No. +ve	Percentage
LAT	280	141	50.4
ELISA	280	172	61.4
Both LAT and ELISA	280	134	47.9
Exam of mutton samples for harboring tissue cysts	134	28	20.9

Table 2: Percentages of *T. gondii* strains in mutton samples harboring tissue cysts

Total isolates		Type I		Type II		Type III		Avirulent strains	
No	%	No	%	No	%	No	%	No	%
28	20.9	3	10.7	14	50	6	21.4	5	17.9

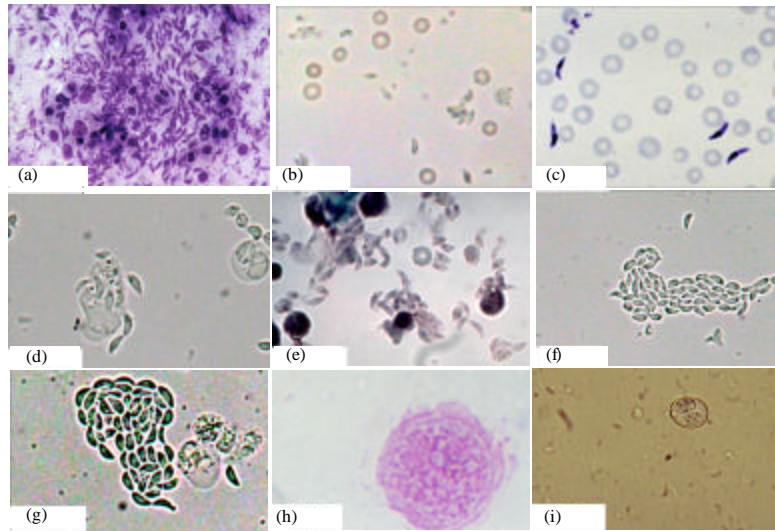


Fig. 1(a-i): Massive number of *T. gondii* Type I (RH) strain free tachyzoites (a) little number of type II and III free tachyzoites, (b, c) high virulent type I tachyzoites liberated from leukocytes, (d) leukocytes were trapping and engulfing tachyzoites, (e) tachyzoites aggregations of type II and III and beginning of tachyzoites-bradyzoites re-conversion, (f, g) ideal type II or III tissue cyst from mice brain (h) and typical *T. gondii* sporulated oocyst (i)

beginning from 7 DPI; Type II (ME49) strains of mildly virulent character, where, the mice lethal dose [ $LD_{50} 10^2$ ], the majority of mice did not persist more than 3 weeks post infection and about 30% persist ill up to 2 month post infection, tissue cysts were clearly demonstrated from 45 DPI and typical and huge numbers of oocysts were shed in cat's feces from 7 DPI for 15-20 days; Type III (PRU) strains of low virulence where, the lethal dose [ $LD_{50} 10^3$ ], the majority of mice could survive but about only 10% persist morbid up to 2 month post infection, tissue cysts were clearly demonstrated from 45 DPI and moderate number of typical oocysts was shed in cat's feces for 7 DPI from 3 DPI and avirulent isolates without any signs of pathogenicity in mice or kittens and few avirulent tissue cysts  $\geq 60$  DPI were demonstrated (Table 3, Fig. 1).

Table 3: Classification of mutton isolates according to bioassay in mice and kittens

Isolates	Type I RH strain	Type II ME49 strain	Type III PRU strain	Avirulent isolates
<b>Mice bioassay</b>				
Lethal dose	LD <sub>100</sub> _1	LD <sub>50</sub> _10 <sup>8</sup>	LD <sub>50</sub> _10 <sup>8</sup>	----
Death time	5 DPI	21/60 DPI	45/60 DPI	-----
Tissue cysts	---	Huge (45DPI)	Moderate (45DPI)	Few (60 DPI)
Brain cysts	----	Huge (60 DPI)	Moderate (60DPI)	---
<b>Kittens bioassay</b>				
Shedding time	7-10 DPI	3-21 DPI	3-10 DPI	---
Shedding duration	few days	Huge= 18 days	Moderate (7 days)	---
Oocysts characters	Atypical	Typical	Typical	---

## DISCUSSION

In the present study, high prevalence (50.4% and 61.4% by LAT and ELISA) of *Toxoplasma gondii* IgG antibodies in the tested sheep sera was regarded as high risk animal group for both public and animals' health, these results agreed with that previously obtained in Egyptian sheep by Shaapan *et al.* (2008) and Barakat *et al.* (2009) using different serological tests and thus high prevalence was referred to the pattern of raising of Egyptian sheep on oocysts dirty unsanitary circumstance as proved by Hassanain *et al.* (2008) who detected high percent of naturally infected Egyptian un-weaned and weaned kittens with *Toxoplasma* (70.6 and 50.8%) and the study also ensure that the sero-negative kittens were better shedders (11.34%) than the sero-positive ones (3.09%).

Theoretically, all sero-positive sheep could harbor tissue cysts; however, in the present study, only 20.9% of the 134 sero-positive pepsin digested mutton samples were found to harbor tissue cysts. Present result is nearly similar to that reported by Belbacha *et al.* (2004) who found tissue cysts in 30% of 50 brains taken from sero-positive Morocco sheep. These results signify that not all sero-positive animals were harboring virulent bradyzoites in their tissues indicating failure of the sero-diagnostic tools to judge all sero-positive animals as a hazard source and these results may be due to the transferred maternal antibodies or immunity against avirulent none cyst forming strains beside the possible incompatibility between carcasses tissue cysts distribution and the taken mutton samples (Owen and Trees, 1999).

The sero-positive mutton samples containing tissue cysts in the present work when biologically assayed in mice and kittens, 10.7, 50, 21.4 and 17.9% were corresponding to type I, II, III and avirulent strains, respectively. Nearly similar results obtained by Zia-Ali *et al.* (2007) who detected *T. gondii* isolates from adult sheep in Iran, 2 isolates were Type II and 2 were Type III and found most stock isolates were avirulent for mice, while few were highly virulent producing acute toxoplasmosis. In present results, the majority (50%) of the examined mutton isolates belonged to type II. These results incompatible with that of Asai *et al.* (1995), who stated that the virulent sheep isolates in Japan were the most isolates and vast majority of type II strains and also with of Dumetre *et al.* (2006), who they found that all the eight *T. gondii* isolates from adult sheep from France were clonal type II.

Moderate prevalence (17.9%) of mutton isolates was confirmed to be avirulent in the current study. On the contrary, Cremers *et al.* (1991), did not find viable bradyzoites of digests of 5 g muscle samples from 40 sero-positive slaughtered sheep and aborted ewes in Denmark. This disparity in results was attributed to that the practical biological demonstration of these avirulent isolates is very difficult because it behave asymptomatic and with only little unpathogenic tissue cyst formation in susceptible survived inoculated mice (Buxton *et al.*, 2007).

Mixed infection in the same mutton sample may be involved more than one type as shown in this study. Support our inspection that; it has recently been reported that multi-genotype infections are common in animals and humans as demonstrated by Aspinall *et al.* (2003), they using high-resolution micro satellite analysis and concluded that the presence of more than one type of parasite in individual human samples might be related to food-borne infection. Although, no cases of mixed human infection have ever been identified by mouse inoculation in vitro culture (Boothroyd and Grigg, 2002) but experimental infection by Type II and III strains could produce different viable progeny in cats (Kong *et al.*, 2003).

## CONCLUSION

In the present study; not all sero-positive sheep are containing the *T. gondii* tissue cysts, determined that sero-diagnostic tool individually failed to judge mutton as a hazard source. The bioassay identified *T. gondii* tissue cysts containing mutton corresponding to the three virulent types, indicating zoonotic impact on public health and directing for avoiding consumption of under cooked meat. The study also analyzed the biological diversity of local Egyptian sheep isolates which is considered as an essential step for further genotyping.

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