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Zoonotic Hazards *T. gondii* Viable Cysts in Ready to Eat Egyptian Meat-Meals

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Abstract: *Toxoplasma gondii*, a meat cyst-forming protozoan, has a potential zoonotic impact sequence to consumption of undercooked meat or chickens harboring bradyzoites. The present study, explores some common Egyptian meat and chicken meals (413 samples), which are still neglected as a possible source that can maximize the incidence of human toxoplasmosis such as sausages, koffta, kebab, shawerma, basterma and offal, plus to chickens were examined for the persistence of viable *T. gondii* bradyzoites through direct microscopical examination of digested meat samples followed by mice viability test, plus using PCR for recognition of *T. gondii* DNA of either viable or unviable bradyzoites. Through the microscopical examination, offals (43.4%), mutton kebabs (42.9%), brain tissue (30.6%) and sausage (26.9%) showed the highest percentage of toxoplasma contamination. While using the mice passage testing, mutton kebabs (29.2%), sausage (21.4%), Core pastrami (18.8%) and brain tissue (18.2%) showed the highest percentage of toxoplasma contamination. From the other side, viability testing showed the highest percentage in sausage (61.8%), mutton kebabs (51.1%), Core pastrami (49.7%) and brain tissue (43.6%). Shawerma, Kofta, luncheon, Superficial pastrami and boiled chickens (house kitchen) were completely negative using microscopical examination, mice passage and viability testing. Finally using PCR, the mutton kebabs (57.1%), offals (50.9%), boiled chickens (42.9%), Barbeque chickens (41.9%) and brain tissue (41.7%) showed the highest percentages of toxoplasma contamination. More attention should be undertaken to these selective Egyptian meat meals as it is proved to be responsible for the high incidence of human toxoplasmosis in Egypt. PCR should be used with the other traditional toxoplasma diagnostic methods as an indicator for the capability of the used cooking or processing method to eliminate the parasite in the meat meals prior for consumption.

Key words: Toxoplasmosis · Egypt · Meat meals · PCR. Viability

INTRODUCTION

Toxoplasma gondii is an obligate intracellular tissue cyst-forming protozoan; the disease is affecting a wide range of different animal species in Egypt including small ruminants [1], large ruminants and swine [2], equines [3], camels [4], rabbits [5] and poultry [6] causing serious economic losses especially in the sheep industry all over the world.

Toxoplasmosis is also a typical meat borne zoonosis, where most human infections occur through the bradyzoite stage that present in the edible meat [7] and exceeding opportunistic human impact sequence to cerebral [8], congenital [9] and ocular toxoplasmosis [10]. Maternal-fetal pass in pregnant women possibly develops abortion, stillbirths, mental retardation, plus neurological sequel of congenital malformations and ocular disorders [11].

Recent ecological and etiological investigation concerning risk factors should be updated to solve the unexplained equation of high sero-prevalence of human toxoplasmosis specially in pregnant women in Egypt (51.4%) as reported by Ibrahim *et al.* [12] using ELISA. Classically, consumption of undercooked meat has been ascribed as the major risk factor, followed by oocysts dusting and placental diffusion. Where, amplifying awareness against undercooked meat minimizes human prevalence world-wide. Therefore, many reports have

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indicated that human toxoplasmosis mainly took place in many localities free from cats [13, 14]. This is in contrast to farm animals that are usually infected by cohabitation with cats [15, 16].

The prevalence of food animals' meat harboring *T. gondii* viable tissue cysts are completely different from country to other according to the socio-economic pattern of housing the final host cats and the human feeding behavior[16]. In cyst, bradyzoites remain viable and infective for as long as the meat is edible [17, 18], Tenter *et al.* [19] and Schlundt *et al*. [20] estimated that the percentage of meat-borne cases was approximately 30 to 63%, depending on eating habits.

Dormant cysts in meat was rendered nonviable when internal temperatures had reached 67°C or freezed at -12°C for 1 day [21], but occasionally some strains may survive deep-freezing [22]. Superficial heating does not kill all bradyzoites [23] and remain infectious in refrigerated carcasses (1-4°C) or minced meat for up to 3 weeks [24], also survive freezing at temperatures between -1 and -8°C for longer than a week [25]. Also, salting was found to be effective at 5% sodium chloride for not less than 8 days [26]. Chilling at (4°C) was found to enhance the preservative time as a storage method saving bradyzoites viable up to12 days [21]. Thus, different types of undercooked processed meats and carcass offal's represent toxoplasma public health hazard for harboring *T.gondii* viable cysts. So, the current study focused on studying the possibilities of some neglected Egyptian meals as a source of human toxoplasmosis. The study design was based on viability test in mice and toxoplasma DNA detection from ready to eat meat and chicken meals.

MATERIALS AND METHODS

Samples Collection: Random samples of ready to eat meat and chickens meals (shawerma, kofta, luncheon, superficial and core parts of pastrami, boiled and barbeque chickens, offal's, brain tissue, sausage and mutton kebabs) were collected from different stores and restaurants of quick meat meals from Cairo and Giza governorates, Egypt, during 2012-2013. The collected samples were regularly examined microscopically followed by mice viability test and Polymerase Chain Reaction (PCR).

Digestion and Microscopical Examination of Samples: Fresh 20 gm from each of the previously mentioned meat and chicken samples were homogenized separately in 100 ml of 2.5% pepsin in PBS and incubated at 37°C for 1.5hr with continuous shaking. Following incubation the large particles were removed by filtering with fine mesh gauze and then microscopically examined at low and high powers [27].

Mice Viability Test: Each sample from examined meat specimens was cut into 10 g portions and ground in a mortar using 5 ml of antibiotic saline solution (physiological saline 0.85% containing 100 U/ml of penicillin and streptomycin and 12.5 U/ml heparin). Approximately 5 g of the suspension were removed and placed into a sterile container. The remainder of the sample was stored in sterile containers at 4°C for the use in mice inoculation [28].

The microscopically confirmed tissue cysts containing samples were biologically bio-assayed via intrapersonal inoculation of toxoplasma sero-negative mice. All over the course of the study, a total number of 260 sero-negative Swiss Webster albino mice obtained from Laboratory Animals House, National Research Center, Egypt, were used; each positive meat or chicken sample of microscopically confirmed tissue cysts was intraperitoneally inoculated into two mice. The animals were inspected daily for ascites that indicate acute toxoplasmosis, followed by signs of tottering gait, hunched appearance, evidence of early emaciation and dehydration. These animals were culled immediately and a sample of peritoneal exudates was removed and inspected for tachyzoites by microscopic examination. Blood samples were exposed to ELISA while tissues from internal organs and brain were collected for tissue cyst demonstration and PCR testing.

The work is approved ethically by the Medical Research Ethics Committee-National Research Centre, Al Buhouth St., Dokki, Giza, Egypt-under registration number 1-2 /0-2-1.2012.

ELISA: Sera were collected from the injected 260 sero-negative mice, either during scarifying symptomatic mice or at 7days post infection in survived non symptomatic ones. The optimum antigen, serum and conjugate concentrations were determined by checkerboard titration and test procedures were carried out according to Lind *et al*. [29]. The cut-off values of optical density (OD) were calculated according to Hillyer *et al*. [30].

DNA Extraction:

Preparation of Specimens and Isolation of DNA for PCR: DNA extraction from meat was performed by GF-1 Tissue DNA Extraction Kit (ViVantis Co., Malaysia). 30 mg of

each meat sample was cut into small pieces with sterile scalpel and was put into 1.5 ml PCR tube followed by washing in PBS, pH 7.4 for three times with pulsed vortexing for 5 seconds. Then the samples were treated following the manufactures instructions. DNA was eluted in 50µL of elution buffer and stored at -20°C to be used for PCR amplification.

PCR Amplification: Primers used for PCR were targeting the repetitive 35-fold B1 gene [31]. The outer primers were only used in this study. The reactions were set up to a final volume of 25 µL containing 2µL of Toxoplasma RH strain DNA as standard control or 10µL of meat specimen DNA, 1 µL of each primer (100 pmol) and 12.5 µL Pyo-Stari™ Fast PCP Master Mix (Fermentas Co., Cat. No. Ko211). PCR was performed in a T-gradiant, Biometra, Germany. 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.

RESULTS

From Table (1) and Figures (1-4), using the microscopical examination, offal (43.4%), mutton kebabs (42.9%), brain tissue (30.6%) and sausage (26.9%) showed the highest percentage of toxoplasma contamination. While using the mice passage testing, mutton kebabs (29.2%), sausage (21.4%), core pastrami (18.8%) and brain tissue (18.2%) showed the highest percentage of toxoplasma contamination. From the other side, viability testing showed the highest percentage of toxoplasma contamination in sausage (61.8%), mutton kebabs (51.1%), Core pastrami (49.7%) and brain tissue (43.6%).While Shawerma, Kofta, luncheon, Superficial pastrami and boiled chickens (house kitchen) were completely negative using microscopical examination, mice passage and viability testing.

Finally from table (1) and figure (4), the results of PCR showed that mutton kebabs (57.1%), offals (50.9%), boiled chickens (42.9%), Barbeque chickens (41.9%) and brain tissue (41.7%) had the highest percentages of toxoplasma contamination whereas superficial pastrami (21.6%) showed the lowest percentage.

It was noted that, Shawerma, Kofta, luncheon, superficial pastrami and boiled chickens showed positive results in PCR although they were negative in all the other diagnostic tests (Fig.5). Also mutton kebab and brain tissue showed high contamination with *T. gondii* in all the used diagnostic methods.

DISCUSSION

Human toxoplasmosis is mainly meat borne zoonoses, the relative importance of the risk factor and the type of meat associated with it varied among different countries [32]. For example, in France and Norway consumption of undercooked lamb was a stronger risk factor than consumption of undercooked pork [33], whereas in Poland it was the consumption of undercooked pork [34]. These findings may reflect differences in eating habits of consumers or different prevalence of infection in meat producing animals in these regions.

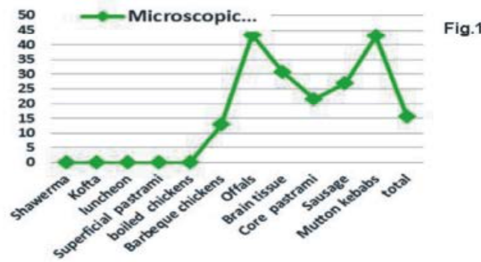
Feeding habits among Egyptian population have been extremely changed during the last years, due to the establishment of restaurants serving quick meat and

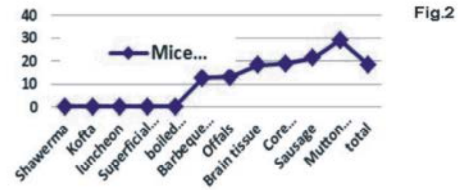
Table 1: Results of different diagnostic for Toxoplasma in meat and chicken meals

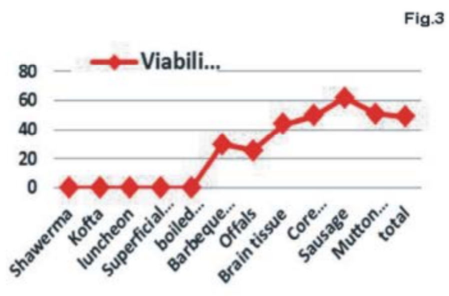
	N	ME Suspected	mice passage	Toxoplasmosis Positive PCR	Viability (%)
Shawerma	44	0	0	13(29.5%)	0
Kofta	65	0	0	22(33.8%)	0
luncheon	35	0	0	11(31.4%)	0
Superficial pastrami	37	0	0	8(21.6%)	0
boiled chickens (house kitchen)	21	0	0	9(42.9%)	0
Barbeque chickens	31	4(12.9%)	1/8(12.5%)	13(41.9%)	29.8
Offals	53	23(43.4%)	6/46(13%)	27(50.9%)	25.5
Brain tissue	36	11(30.6%)	4/22(18.2%)	15(41.7%)	43.6
Core pastrami	37	8(21.6%)	3/16(18.8%)	14(37.8%)	49.7
Sausage	26	7(26.9%)	3/14(21.4%)	9(34.6%)	61.8
Mutton kebabs	28	12(42.9%)	7/24(29.2%)	16(57.1%)	51.1
Total	413	65(15.7%)	24/130(18.5%)	157 (38%)	48.7

We were excluding the UV radiation due to inapplicable use.

Viability (%) = Mice passage (%) / +ve PCR (%)







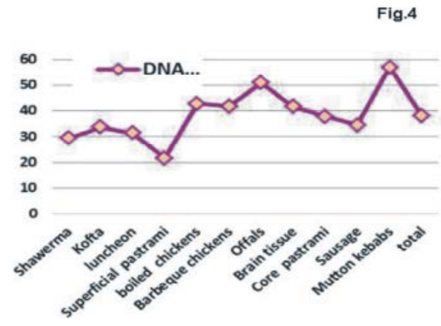


Fig 1: Showing the percentage of toxoplasmosis positive Egyptian meat and chicken meals using microscopic examination.

Fig 2: Showing the percentage of toxoplasmosis positive Egyptian meat and chicken meals using mice passage testing

Fig 3: Showing the percentage of toxoplasmosis positive Egyptian meat and chicken meals using viability testing

Fig 4: Showing the percentage of toxoplasmosis positive Egyptian meat and chicken meals using PCR

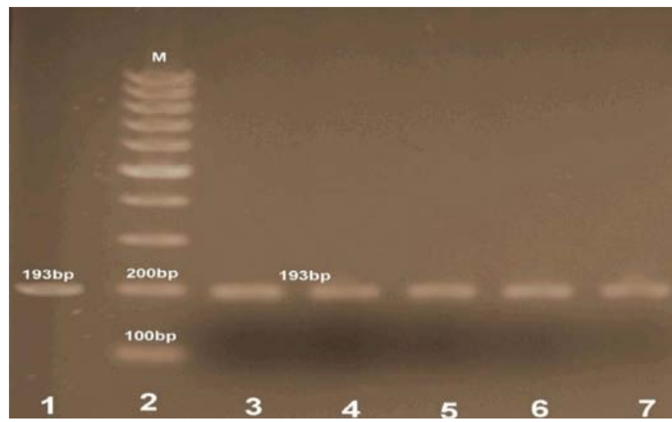


Fig 5: Detection of *T.gondii* in meat samples by PCR, M: Mol. wt. marker (100 bp ladder); 1: Positve control; 3-7: Positive PCR *T. gondii* samples at 193 bp.

chicken meals that may be insufficiently cooked, possible harboring viable *T. gondii* tissue cysts. Thus, the high Egyptian prevalence could be related to undercooked meat rather than oocyst exposure [35]. Usually manufacturing meat products depends on inefficient heating or salting, also, ineffective chilling or freezing are the common tools used for storage.

In the present study, the practical confirmation of the persistence of viable *T. gondii* bradyzoites in ready to eat marketing meat was done through direct microscopical examination of digested samples followed by mice passage test, viability testing plus using PCR for recognition *T. gondii* DNA of either viable or unviable bradyzoites.

It was noted that, shawerma, kofta, luncheon, superficial pastrami and boiled chickens showed positive results in PCR although they were negative in all the other diagnostic tests. Also mutton kebab and brain tissue showed high infection with *T. gondii* in all the used diagnostic methods. All case-control studies have identified the consumption of mutton/lamb meat as a highly significant risk factor for contracting *T. gondii* infection in pregnant women [36].

The high seroprevalence in sheep and cattle and poultry is connected with the high contamination of fresh meat and meat products. Meat from breeding animals is usually processed (such as sausages shawerma, kofta, luncheon, superficial pastrami, mutton kebabs salami, etc.) and processing kills or reduces *T. gondii* as confirmed by Dubey [37].

The sensitivity of bioassay is good, since it allows the detection of 1 cyst in 100 grams of tissue as reported by Dubey *et al*. [38]. However, mouse bioassay requires use of live animals, which are not desirable for screening large numbers of samples from an animal ethics point of view, are time-consuming and not suitable for slaughterhouse testing [39].

In the present study, 85.3% of the microscopically suspected *T.gondii* bradyzoites samples didn't successes in mice passage, possibly due to infection with other tissue cyst forming protozoa, low parasite load, plus possible bacterial or fungal contamination of the collected samples as reported by ELfadaly [35].

The high percentage of contamination with *T. gondii* in boiled chickens (PCR) and Barbeque chickens (all tests) may be attributed to the high positive percentage of infection in the Egyptian chickens (40.4%) as reported by Ibrahim *et al.* [6]. In Egypt, the main risk factor associated with chicken seropositivity might be the contact with soil-harboring oocysts from homeless cats [12]. Poultry is considered a good indicator of environmental contamination by Toxoplasma oocysts as reported by Lehmann *et al.* [40]. However, the processing and handling procedures didn't completely inactivate the organisms prior to sale to consumers as shown in the viability of the parasite in the barbeque chickens (29.8%).

In the present study; confirmation of the persistence of viable *T. gondii* bradyzoites in ready to eat marketing meat was done by using PCR, via amplification of the B1 gene which is highly conserved across strains of *T. gondii* and has a high copy number in the *T. gondii* genome [31, 41]. The high sensitivity and specificity of PCR allows the recovery of parasite DNA at low concentration even exposed to different treatment of freezing, cooking and salting conditions plus to altered pH [42]. Based on the efficiency shown by PCR in the recovery of *T. gondii* DNA we succeeded in identifying *T. gondii* DNA from157/413(38%) of ready-to-eat meat and chicken samples. Basterma which is treated mainly by superficial salting gave relatively low positive percentage in PCR (21.6%).The high salt content of some cured meats like Basterma limited sensitivity of the PCR assay by inhibition of the polymerase enzyme [43]. Shawerma which is treated mainly by superficial heating gave higher PCR positive percentage (29.5%). Nevertheless, Mutton kebabs gave the highest percentage (57.1. %.) of DNA isolation.

The higher positivity obtained by PCR (38%) in comparison with the other methods may be because PCR demonstrates the presence of *T. gondii* and not the presence of viable parasites capable of initiating a human infection as agreed by Aspinall *et al* [44] and disagreed by Bellete *et al*. [45] and Garcia *et al*. [46].

Canada *et al*. [47] reported that the high seropositivity reported in some studies may not correlate with presence of parasites in the meat and, the isolation of infective tissue cysts from beef is rarely reported. This was not the case in our study as the high seroprevalence of infection in buffaloes and cattle in Egypt as reported previously by Hassanain *et al*. [2] was parallel with the high contamination with toxoplasmosis in meat samples used in our study. Concerning the viability results, shawerma, kofta, luncheon and superficial pastrami samples were completely eliminated from the parasite may be due to that these Egyptian meals were exposed to one or more of the following treatments: long freezing (during importation), heating and salting, while other meat meals such as offals, brain tissue, core pastrami, sausage and mutton kebabs still have considerable viable cysts, this may be due to the undercooking(insufficient heating) or inefficient salting. This came in agreement with ELfadaly [35] who decided that only heating at 70°C for 4 min, freezing at - 20°C for 4 days, chilling at 4 °C for 14 days and salting using 5% sodium chloride salt solution for 8 days completely destroy the *T. gondii* tissue cysts confirmed by complete negative microscopic examination and mice viability test.

The present study threw the light on the high risk of consumption of ready- to-eat meat meals that are not exposed to enough heating or salting. This may be responsible for the high incidence of human toxoplasmosis in Egypt.

CONCLUSION

Improved surveillance is needed to better estimate the true incidence of foodborne toxoplasmosis in Egypt. More studies are required to estimate the effect of different environmental conditions on the viability of the parasite in different Egyptian meat meals.

More attention should be undertaken to these selective Egyptian meat meals such as sausage, mutton kebabs, core pastrami and brain tissue as it is proved to be responsible for the high incidence of human toxoplasmosis in Egypt.

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