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Original Research Article

Bio-typing of *Toxoplasma gondii* isolates from complicated pregnant Egyptian women

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Abstract

***Toxoplasma gondii*; is one of the most prevalent abortificiant human protozoan inducing complicated pregnancy, has usual colonial population of three virulent types; I, II, and III; with dissimilar ecological and zoonotic modes of transmission. The objective of the present study is directed towards the biological typescript of *T. gondii* local Egyptian isolates which collected from complicated pregnant women by using mice passage and kittens. The current study deals with isolation and biological characterization of *T. gondii* isolates from complicated pregnant Egyptian women. Placenta land or fetal tissue samples were collected from one hundred and six women attending the labour ward of (Beni-Suef General Hospital and Kasr Al-Aini Hospital-Egypt), which were suffering from complicated pregnancy. The study includes G1, G2, G3 and G4 which symbolized the abortion corresponding to the 1st, 2nd and 3rd trimesters with congenital anomalies. The fresh placental tissue samples were microscopically examined after pepsin digestion; the confirmed bradyzoites containing samples were bio-assayed by intraperitoneal (IP) passage in mice as intermediate host model to conclude the mice Lethal Dose [LD] and the tissue cyst forming characters. Wherever, the isolates successive lypass in mice were bio-assayed by oral inoculation in a toxoplasma free un-weaned kittens for confirmation the oocysts shedding properties. Accordingly, different isolates were biologically typescript to one of type I, II or III. The results showed that seven local isolates (6.6%) separated from complicated pregnant cases and were biologically characterized in mice and kittens as cystogenictype-II, with ecological conclusion of transmission of infection mainly through insufficient cooked meat harboring viable tissue cyst.**

Keywords: Complicated pregnancy, mice and kittens' bioassay, Egyptian isolates, *Toxoplasma gondii*

INTRODUCTION

Toxoplasma gondii is a latent opportunistic zoonotic protozoan; signify one of the most prevalent abortificiant human pathogen, with varies complicated pregnancy (Sibley et al., 2009). It exists in three infective stages; the acute tachyzoite stage responsible for the materno-fetal pass, and maybe transmitted to latent tissue cyst containing bradyzoites in somatic cells including uterine and placental tissues. Oocyst is the sexual stage developed only in the feline's gut; and human's primary infection mainly occurs through consumption of either inadequately cooked meat harboring viable brady-zoites or oocysts contaminated food or water, pass to the

placental diffusion (Tenter et al., 2000).

Only one species of the genus *Toxoplasma* has been observed with three clonal lineages (I, II, III) reference to their varied virulence and pathogenicity in mice. Type I strain being uniformly acute lethal non-cyst forming in outbreed mice (LD1_100), and lead to death of mice less than10 days after inoculation of < 10 tachyzoites; in contrast, types II and III are considerably less virulent cyst-forming strains with neurological symptoms (Howe and Sibley, 1995). Latent cystogenic strains (cyst-forming) types II and III are equally predominant (75%) in human toxoplasmosis (Zhou et al., 2004). Although type I strains are relatively rare in animals but may be more likely to cause severe human congenital toxoplasmosis, toxoplasmic abortion and ocular disease (Chemla et al., 2002). According to such biological diversity, we could expect diverse materno-fetal courses and different complicated pregnancy corresponding to the different types.

Mice bioassay of isolated organism from amniotic fluid, placenta and fetal blood of higher sensitivity and specificity, and considered a standard test to detect *T. gondii* in tissues (Homan et al., 2000).

The objective of the present study deals with biological typescript of *T. Gondii* local Egyptian isolates which collected from complicated pregnant women by using mice passage and kittens.

PATIENTS, MATERIALS AND METHODS

Study population

A cross sectional observational hospital-based study was conducted on one hundred and six females presenting with complicated pregnancy, with age range between 18 and 40 years. These patients were selected from the labour ward of (Beni-Suef General Hospital and Kasr El-Eini Hospital) during the period from July 2011 to October 2012. All studied cases were subjected to complete history taking using a questionnaire sheet.

The study population was classified according to the obstetric history of pregnancy into four groups:

- Group 1; females presenting with abortion in the 1st trimester [≤ 12 weeks of gestation] (No.=56)
- Group 2; females presenting with abortion in the 2nd trimester [13- 26 weeks of gestation] (No.=30)
- Group 3; females presenting with intrauterine fetal death [27 weeks of gestation to full term] (No.=15)
- Group 4; females gave birth to babies with congenital anomalies (No.=5)

Exclusion criteria included recent trauma, positive consanguinity, systemic diseases, Rh incompatibility and other risk factors suggestive of complicated pregnancy. The study was approved by Medical Research Ethics Committee of the National Research Center, and written consent was taken regarding this issue prior to the sample collection.

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

Toxoplasma gondii reference (control) strains

In the present study the three strains RH, ME-49 and Prugniaud (PRU) were secured in Zoonotic Diseases Department, National Research Center, Egypt, used as type's control strains, representing types I, II and III with the following lethal doses: LD100 _1, LD50 _ 10^2, LD50 _ 10^3 respectively in outbreed mice. The RH strain tachyzoites were maintained through successive intraperitoneal tachyzoites - tachyzoites passages in mice every 3 days, the obtained tachyzoites from mice peritoneal fluid of infected 3 days earlier were used for intraperitoneal infection after counting and dilution as necessary (10^5–10^6 factor dilution). The ME49 and PRU strains (cystogenic strains) were maintained by an oral inoculation of cysts or oocysts in mice for 3 months. Under sterile conditions, brains from infected mice were homogenized in a 20-ml potter's tube, and brain cysts were counted and diluted as necessary (5–20factor dilution), ready for oral or intraperitoneal injection for continuous passage in mice.

Collection of tissue samples

For each studied case, tissue samples were collected. For group 1: about 50 gm of the conception products were obtained by dilatation and curettage (D and C) (Dubey, 1998). For group 2, 3 and 4: about 50 gm portions from placentae were collected (Dubey, 1998), washed three times in phosphate buffered saline (PBS), tissue samples were kept at the refrigerator (4C) till being digested and microscopically examined.

Digestion and microscopically examination of the collected samples

Tissue samples from each case were cut into small pieces (1-2 cm), mixed with an equal volume of acid pepsin solution to release bradyzoites from tissue cyst according to(Dubey et al., 1998 and Dubey et al., 2003b).

Biological bio-assay in mice and kittens

The microscopically suspected tissue cysts containing samples were biologically bio-assayed in both toxoplasma sero-negative mice and kittens, the biological typing was

based on intrapretonial inoculated toxoplasma sero-negative mice as intermediate host model for determine both the lethal dose [LD] and tissue cyst forming characters. Besides, the bioassay by oral inoculation of succeeded isolate in sero-negative kittens as final host for determines the oocysts shedding class (Montoya, 2002).

Viability bio-assay in mice

Sero-negative Swiss Webster albino mice were regularly obtained from Laboratory Animals House, National Research Center, Egypt, were used, Each sample of microscopically suspected bradyzoites was intraperitoneally inoculated into two mice identical to those described by Mercier et al. (2010). The animals were inspected daily for signs of a febrile response that may indicate acute toxoplasmosis, tottering gait, hunched appearance alongside evidence of early emaciation and dehydration) were immediately sacrificed and a sample of peritoneal exudates removed and inspected for tachyzoites by microscopic examination Dubey et al. (2003a). Tissues from internal organs and brain were collected for tissue cyst demonstration. Tissue imprints of died mice were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 45 post-inoculation (p.i.), their brains were examined microscopically for tissue cysts, and a portion of the brain was frozen for DNA extraction. Mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were demonstrable in their tissues. Parasite load was determined by calculating number of tissue cysts / brain multiplying in number of bradyzoites/ cysts. Brain parasite load was then counted in average one gm brain using haemocytometer according to (Elfadaly, 2007).

Viability bioassay in kittens

A total number of twenty three sero-negative kittens was used; 6kittens 2 ones each were inoculated with the three control virulent types I, II and III. Also, 14 kittens 2 ones each were inoculated with the succeeded women isolates; while the three remaining kittens still un-inoculated control ones. Laboratory kittens were fed tissues or orally inoculated the peritoneal washes of mice confirmed to harboring tachyzoites or bradyzoites. Kittens were housing in separate cages, feed on toxoplasma free supplement and their fecal matter were microscopically examined daily up to 30 (DPI) by salt or sugar flotation technique for oocysts determination. Positive samples containing *T. gondii* oocysts were purified from fecal debris by centrifugation for 5 min at 1800 r.p.m and then purified samples were subjected to cesium chloride (CsCl) solution according to (Dubey and Beattie, 1988 and Dumètre and Dardé 2004). Oocyst characters concerning their numbers, pre-patent periods and shedding courses were monitored for each isolates. Then purified samples were stored at [-20°C] according to Lindsay et al., (2002)

Isolates biological typing according to (Villena et al., 2004; Dardé 2004; Elfadaly, 2007; Mercier et al., 2010)

Virulent isolates will represent by significant pathogenicity in experimental injected sero-negative mice, where the lethal dose (LD) and tissue cyst characters will be determined. While oocysts shedding properties were determine by orally inoculated sero-negative kittens. Biological typing of isolates will done according to (Dardé, 2004), who classified the vast majority of isolates studied until now belong to only 3 clonally lineages designated types I, II and III based on the proprieties of data monitoring for each isolates that referred to the used control isolates (Table 2), represented by LD, ability of tissue cyst formation and oocysts characters. While a virulent isolates unpathogenic in experimental injected mice. Biological typing of virulent isolates was determined by mice clinical signs together with cat shedding skills and reference to the three types I, II, III.

RESULTS AND DISCUSSSION

The present study concerns the ecology and zoonotic impact through biological typescript of *T.gondii* virulent strains which have varied communication manners, and associated with one hundred and six Egyptian women suffering complicated pregnancy at different trimesters. Although isolation of *T. gondii* tissue cysts from woman placenta is so difficult (Ferreira et al., 2011), but success isolation was strongly correlates with fetal infection (Fricker-Hidalgo et al., 2007), and the present study revealed (6.6%) isolates are succeeded in mice passage, where only 10% pepsin digested placental samples were succeeded in mice passage harboring cysts. While, the remaining 90 % were identified tissue cysts free women .The results signify that not all women were harboring placental bradyzoites; possibly due to incompatibility between placental cysts distribution and the taken tissue samples or may be sequence to avirulant none cyst forming strains.

In the present study; high incidence (6.6%) of toxoplasmic abortion was detected in women suffering complicated pregnancy possibly sequence to primary infection during pregnancy. But also, vague dynamics of intrinsic latent infection habitually opportunist and motivate the dormant bradyzoites to tachyzoites re-conversion (Roberts et al., 2001), maybe related to hormonal factor equivalent to gestation period sex steroids hormonal sift, where sharp exploits of progesterone and estrogen levels inspire materno-fetal

Table 1. Variations of positive microscopic examination & bioassay of suspected samples in different groups

Group	Positive microscopic ex. No. (%)	Positive mice bioassay No. (%)	Positive kittens bioassay No. (%)
G1	20/56 (35.7)	2/20 (10)	2/20 (10)
G2	8/30 (26.7)	1/8 (12.5)	1/8 (12.5)
G3	6/15 (40)	3/6 (50)	3/6 (50)
G4	2/5 (40)	1/2 (50)	1/2 (50)
Total	36/106 (34)	7/106 (6.6)	7/106 (6.6)

G1 = abortion in the 1st trimester, **G2** = abortion in the 2nd trimester, **G3** = abortion in the 3rd trimester (IUFD), **G4=** deliverers of babies with congenital anomalies.

Table 2. Biological typing of T.gondii local Egyptian isolates from women according to LD, tissue cyst characters and oocysts shedding reference to Toxoplasma strains (I,II,III) in mice & kittens according to Dardé (2004).

MICE BIO-ASSAY	**Type I RH strain**	**Type II ME49 strain**	**Type III PRU strain**
LD	LD100_1	LD50 _ 10^2	LD50 _ 10^3
Death time	5 DPI	21/60 DPI	45/60 DPI
Tissue cysts	-	Huge ≥ 45 DPI	moderate ≥ 45 DPI
Brain cysts	-	Huge ≥ 60 DPI	moderate ≥ 60 DPI
KITTINS BIO-ASSAY			
Shedding time	7-10 DPI	3-21 DPI	3-10 DPI
Oocysts amount	None or Very few	Huge≥18 days	Moderate /seven days
Characters	Atypical	typical	typical
NO of corresponding women isolates	0	7	0

Type I
Highly virulent strain for mice highly disseminated with rapid death of 100% of mice even with one tachyzoite (LD1-100), difficult to form tissue cyst, and shed none or very few atypical oocysts at 7-10 DPI. Rarely isolated (mainly from sever human congenital & ocular infections). RH strain represents the typical reference of type I.
Type II
Moderately virulent strain for mice, highly produce tissue cysts and shed a huge typical oocysts at 3-21 DPI. The most commonly isolated (mainly from aborted and AIDS human cases).ME49 strains represent the reference control of type II.
Type III
Mildly virulent for mice, moderately produce tissue cysts, and shed a moderate /seven days typical oocysts at 3-10 DPI.mainly isolated from meat producing & wild animals.VEG strains represent the reference control of type III

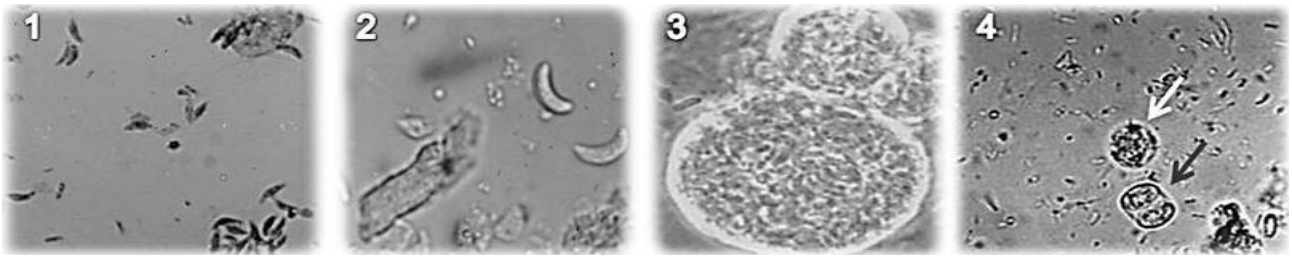

Figure 1. Slid -1: Freeunstained *T. gondii*tachyzoites of RH strain= X400. Slid-2: Unstainedfree bradyzoitesof digested placental tissues= X1000.Slid-3: typical mice brain cyst of succeed passage women isolates X600.Slid-4: Cat shed typical (9 to 14 *u*m) un-sporulated (white arrow) and sporulated (black arrow) in cesium chloride solution from the collected kittens fecal maters inoculated by succeed mice passage isolates X600.

diffusion in pregnant rats (Elfadaly et al.,2012). Also, the opportunistic recurrent infection may be due to temporary gravidity hyperglycemia, where *T.gondii* parasite load elevation was confirmed in diabetic rats as latent opportunistic character (Hassanain et al., 2014).Moreover, the opportunistic relapse possibly series to anti-inflammatory corticosteroids therapy during pregnancy (Elfadaly et al.,2015).

The current study was confirmed the highest percent (52.8) of aborted women was in their 1st trimester with consequence decline in the 2nd and 3rd trimesters (28.3 and 14.2) respectively. While only (4.7) were deliveries babies with congenital anomalies (Figure 1).Wang et al. (2003) and Wyatt et al. (2005) insured this concept where spontaneous abortion was detected in the first trimester and frequency decreases with increasing gestational age.

Few data concerning biological diversity of *T.gondii*are available. The current study of ecological and epidemiological impact, identified type II as adaptive isolates inducing women placental cysts, based on the biological characters in both mice and kittens, and coincide with what's reported that cystogenic strains, mildly virulent in mice, are the most commonly isolates from different aspects of congenital and latent human toxoplasmosis (Robert-Gangneux and Dardé, 2012). However, the cystogenic type II is the most prevalent in animals' meat and were creating oocysts in shedder cats; reflect the high possibility of women in this study to be infected through under cooked meat or environmental oocyst. Also, type II was definite the most prevalent strain in Egyptian mutton (Hassanain et al., 2011), and in Egyptian free-range chickens (Dubey et al., 2003a), the concept was insure through the extra alteration of feeding behaviors among Egyptian consumers through developing of frequent restaurants serving undercooked fast meat meals.

Limited analyses in the USA also supported the overall trend that type II strains are the most common in congenital toxoplasmosis (Howe and Sibley, 1995). Similarly reported in France (Howe et al., 1997andAjzenberg et al., 2002)and also, in Poland (Nowakowska et al., 2006). In a further report from Iran, revealed that were mostly of type II (Asgari et al., 2013). In contrast, in South America, type I was mostly implicated in severe cases of congenital toxoplasmosis (Ferreira et al., 2006and Gallego et al., 2006) and in Saudi Arabia causing (Abd El-Aal et al., 2010). These different results may be explained by that the parasite strains show strong correlations with geographical boundaries and thus vary dramatically in different localities (Weiss and Kim, 2007). Mixed infection of more than one strain may be involved, but higher virulent types hidden the biological properties of less virulent ones, consequently the parasite isolation process could results in a single strain even if several strains were found in the inoculums (Aspinall et al., 2003; Hassanain et al., 2011).

Conflict of Interest

No conflict of interest

CONCLUSION

We concluded that Biological typescript of *T.gondii* Egyptian isolates collected from complicated pregnant women revealed cystogenic type-II strains; reflect high suspicion of catching infection via consumption of underdone meat harboring bradyzoites.

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