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Bioassay of *Toxoplasma Gondii* from Apparently Healthy Pigs Slaughtered in Addis Ababa Abattoir, Ethiopia

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Abstract

Toxoplasmosis is an infectious disease caused by the protozoan parasite *Toxoplasma gondii* that infects a wide range of animals, including man and birds. The objective of the study was to isolate *T. gondii* in pigs slaughtered at the Addis Ababa abattoir. A total of 290 sera were examined for *T. gondii* antibodies (IgG) using the Direct Agglutination Test (DAT) of which 111 pigs (38.28%; 95% confidence interval [CI]: 32.65%-44.14%) were seropositive. Hearts of fifty seropositive pigs and two pooled pig heart samples (portion of 4 pig hearts per pool) were used for the study. For the isolation of *T. gondii*, 50 g heart tissue was ground, digested with acid-pepsin and inoculated intraperitoneally into mice (5 mice/sample). Viable *T. gondii* was isolated from hearts of 24 of 50 (48%; 95% CI: 33.7%-62.6%) seropositive pigs and from the two pooled seronegative pig hearts. All isolates were asymptomatic except one isolate from Bishoftu which was lethal for mice on day 28 post-inoculation. There was a perfect agreement (k=0.85) between DAT and microscopic cyst detection in mice. The study suggests that pork containing viable *T. gondii* is released onto markets for consumers. Therefore, enforcing hygienic measures, public education, provision of rendering facilities and further studies deserve consideration. This is the first report of isolation of viable *T. gondii* from pigs in Ethiopia.

Keywords: Bioassay; Pig; *Toxoplasma gondii*; Addis ababa; DAT

Introduction

Toxoplasmosis is an infectious disease caused by the protozoan parasite *Toxoplasma gondii* that infects a wide range of animals, including man and birds. Members of the cat family, most importantly the domestic cat, play a crucial role in the epidemiology of toxoplasmosis as definitive hosts through shedding of oocysts [1,2]. All warm blooded animals and humans serve as intermediate hosts [3,4].

Among livestock, pigs, sheep and goats are more susceptible to *T. gondii* than cattle and chicken. Infection with *T. gondii* occur when pigs, and other animals, accidentally ingest oocysts in soil or water, or eat tissues of infected rodents, wildlife, or meat containing tissue cysts [2,5,6]. This parasite cause abortion, still birth, neonatal death and fetal mummification in female pigs, sheep and goats infected for the first time during pregnancy, leading to significant economic losses to farmers [1,5]. Toxoplasmosis is a zoonotic disease with a high impact on public health [7]. Human infections may go unnoticed or may cause various signs and symptoms depending on the patient's immune and general health status [8,9]. Seroepidemiological studies have demonstrated the huge impact of pork contaminated with tissue cysts on the transmission of toxoplasmosis to humans [10]. It is also known that a single pig intended for human consumption contaminated with cysts in muscle tissue is capable of transmitting the infection between 200 and 400 individuals [11].

Due to the importance of *T. gondii* for animals and public health, and the absence of studies on isolation of the causative organism from pigs in Ethiopia, the present study was undertaken with the objectives of isolation of *T. gondii* from pigs slaughtered for human consumption at Addis Ababa abattoir by bioassay in mice.

Material and Methods

Study area

The study was conducted at the Addis Ababa abattoir. Addis Ababa lies in the central highlands of Ethiopia at an altitude of 2,200 to 3,300 meters above sea level (masl). The average annual temperature is 21°C with relative humidity varying between 70% to 80% during the rainy season and 40% to 50% during the dry season. The area receives an average of 1800 mm rain annually [12]. Addis Ababa abattoir slaughters on average 25 pigs once a week in the afternoon. At the abattoir carcasses stay hanging at the end of the slaughter line until distributed during the next morning without treatment (salting or freezing) to owners for selling to various Addis Ababa supermarkets [13].

Study design, sample collection and transportation

The study was carried out on apparently healthy pigs slaughtered at Addis Ababa abattoir. Pigs of both sex, age groups [young (<12 months) and adult (≥ 12 months)] and originating mainly from Addis

Ababa (Legetafo, Kolfe Keraniho, Akaki Kaliti and Tatek), Bishoftu and Adama were considered for the study. Age was determined by observation of teeth eruptions [14]. Systematic random sampling was carried out to sample blood and heart from a total of 290 pigs. The blood and heart samples were labelled and immediately transported in ice packs to the Ethio-Belgium VLIR project laboratory of College of Veterinary Medicine and Agriculture, Addis Ababa University, Debre Zeit, Ethiopia, for subsequent serological examination and bioassay of *T. gondii*. Sera were separated by centrifugation of the tubes at 3200 RPM for 10 minutes. Then, sera were transferred to other eppendorf tubes and kept in a freezer until serologically assayed. The heart samples were maintained under refrigeration until the results of serological tests were known (24-48 hrs). Random sampling of 50 seropositive pig hearts and 2 pooled heart samples from seronegative pigs were considered for bioassay.

Serology and bioassay of heart tissues of pigs for *T. gondii* in mice

Toxoplasma gondii-specific IgG antibodies in pig and mice sera were detected by direct agglutination tests (DAT) (Toxo screen DA, biomerieux®, France) following the procedure described by the manufacturer of the kit as described previously [15]. Briefly, sera were assayed at a screening dilution of 1:40 and 1:4000. A titer of 1:40 or 1:4000 or both was considered indicative of *T. gondii* exposure. Sedimentation of antigen at the bottom of the well and clear agglutination above half of the well at either dilution were recorded as negative and positive results respectively. DAT positive samples were titrated to know the endpoint titer. Positive and negative controls were included in each test. Bioassay was done according to the description of Dubey [2]. Briefly, each heart was homogenized in saline (0.85% NaCl), mixed with acidic pepsin, and incubated in a water bath for 60 minutes at 37°C. The homogenate was filtered through two layers of gauze, centrifuged, sediment neutralized with sodium bicarbonate and mixed with antibiotic solution (1000 IU of penicillin and 100 µg streptomycin per ml of sterile saline). For every DAT positive pig heart and two pooled pig heart samples (portion of 4 pig hearts per pool), five female Swiss mice (25–35 g each) housed in the same cage were intraperitonealy (i.p) inoculated with the homogenate (1 ml per mouse). Inoculated mice were monitored for 2 months after inoculation and the information on numbers of survivors, dead, day of death, symptoms and weight was recorded. Survivors were bled on day 60 post inoculation (PI). The mice used were *T. gondii* seronegative female Swiss Albino mice, obtained from the animal facility of the National Veterinary Institute, Debre Zeit, Ethiopia. Non-infected mice (n=5) were kept separately as negative controls. The mice were given commercial pelleted feed and municipal chlorinated water ad libitum. *Toxoplasma gondii* isolates were classified as virulent based on mortality of mice within four weeks of infection (i.e. without knowledge of infective dose) [16].

Detection and quantification of cysts from mouse brain

Two months after i.p. inoculation, the brain from surviving mice were removed by sagittal dissection after euthanasia with di-ethyl either. Each brain was homogenized in 1 ml PBS (pH 7.2) using a mortar and pestle. The number of cysts in five aliquots of each 10 µl was counted under a light microscope with a 100X magnification. The total number of cysts in the brain of each mouse was determined by converting the sum of cysts in 50 µl to the whole volume of the brain homogenates [2,17,18]. A bioassay was considered positive if at least one *T. gondii* cyst was detected in any of the five inoculated mice. Brain homogenate that remained from microscopic examination were kept frozen for future DNA extraction and genetic characterization of the *T. gondii* isolates.

Data analysis

Data generated were recorded and coded using Microsoft Excel and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). Descriptive statistics, such as percentage, mean, variance, etc. were used to summarize the data. The rate of cyst isolation in mice was compared with explanatory variables (titre of pig and mean weight difference of mice using the Chi-squared test. Kappa index or agreement between microscopic cyst detection and DAT were calculated according to Dohoo et al. [19]. The 95% confidence interval and a significance level of α=0.05 were used.

Ethical issues

This research project was approved by the College of Veterinary Medicine and Agriculture, Addis Ababa University. Permission form animal owners were also obtained prior sampling. All efforts were made to minimize animal suffering during the course of the study.

Results

Anti-*Toxoplama gondii* IgG antibodies was detected from 38.28% (95% CI: 32.65%-44.14%) of the pigs examined (111/290). Viable *T. gondii* was isolated from hearts of 24 of 50 (48%) [95% CI: 35.7%-62.6%] seropositive pigs. In addition, viable *T. gondii* was also isolated from the two pooled pig tissue samples with DAT negative result. All mice were asymptomatic during the follow up period except one virulent isolate from pig originating from Bishoftu, which killed mice on day 28 post inoculation. Twenty-nine mice died within 3 days of inoculation. Detailed results are depicted in Table 1.

Origin	Pig No	DAT titer of seropositive pigs	Bioassay in mice			
			No. seropositive /No. exam	Cyst pos /No. exam	No. died/No. inoculated	Days of death PI (No. of mice)
Addis Ababa	AA45	60	0/2	0/2	3/5	2(3)
	AA66	6000	5/5	5/5	0/5	survived
	AA67	1620	2/5	3/5	0/5	survived
	AA68	6000	2/4	1/4	1/5	3(1)
	AA69	6000	0/4	0/4	1/5	4(1)
	AA70	1620	1/5	1/5	0/5	survived

	AA71	6000	2/5	2/5	0/5	survived
	AA72	162000	5/5	5/5	0/5	survived
	AA77	1620	0/4	0/4	1/5	6(1)
	AA78	6000	0/5	0/5	0/5	survived
	AA83	162000	0/5	0/5	0/5	survived
	AA100	1620	0/4	0/4	1/5	2(1)
	AA101	1620	1/4	1/4	1/5	2(1)
	AA104	1620	0/5	2/5	0/5	survived
	AA105	1620	0/5	0/5	0/5	survived
	AA155	6000	1/5	1/5	0/5	survived
	AA156	1620	1/5	1/5	0/5	survived
	AA158	1620	1/5	1/5	0/5	survived
	AA160	1620	0/3	0/3	2/5	1(2)
	AA169	1620	0/4	0/4	1/5	1(1)
	AA180	1620	0/5	0/5	0/5	survived
	AA219	6000	0/5	1/5	0/5	survived
	AA220	1620	1/5	1/5	0/5	survived
	AA222	1620	2/5	2/5	0/5	survived
	AA225	1620	1/5	1/5	0/5	survived
Addis Ababa	AA226	1620	1/5	1/5	0/5	survived
	AA249	1620	0/4	0/4	1/5	3(1)
	AA250	1620	0/5	0/5	0/5	survived
	AA252	540	0/5	0/5	0/5	survived
	AA254	540	0/5	0/5	0/5	survived
	AA255	540	0/5	0/5	0/5	survived
	AA274	1620	0/5	0/5	0/5	survived
	AA275	60	3/5	3/5	0/5	survived
	AA279	6000	1/3	1/3	2/5	3(2)
Adama area	AA112	1620	0/5	0/5	0/5	survived
	AA113	1620	0/4	0/4	1/5	2(1)
	AA117	18000	2/2	2/2	3/5	2(1), 3(2)
	AA118	6000	0/3	0/3	2/5	2(2)
	AA119	6000	1/3	0/3	2/5	4(2)
	AA130	6000	1/4	1/4	1/5	2(1)
	AA236	1620	0/5	0/5	0/5	survived
	AA249	1620	0/4	0/5	1/5	2(1)
	AA261	1620	2/5	0/5	0/5	survived
	AA263	1620	1/5	2/5	0/5	survived
	AA268	180	0/5	0/5	0/5	survived
Bishoftu	AA42	18000	1/3	1/3	2/5	2(1)
	AA146	1620	0/5	0/5	0/5	survived
	AA256	60	0/5	0/5	0/5	survived
	AA270	60	1/5	1/5	0/5	survived
	ALP2	1620	1/1	1/1	4/5	28(3), 29(1)
Others	P1	-	1/1	1/1	4/5	2(2), 3(2)
	P2	-	2/5	2/5	0/5	survived

Table 1: Summary of isolation of T. gondii from pigs in study area. Others=pooled samples, two pigs hearts in each pool.

Predictors of presence of brain cyst in mice

Among the cyst positive mice, the mean ± standard deviation [SD] of cyst count per mice brain was 157.2 ± 188.96 (range: 29–686). There was significant association (P<0.05) between antibody titres in pigs and the per cent isolation in mice (34.4% at a titre of >6000 vs 13.6% at titres of ≤ 1620). There was also significant (P<0.05) association between demonstration of tissue cysts and mean weight change of mice in that those mice with a mean weight difference of <7.1 g tend to be more cyst positive (25.8%) than those with a mean weight difference ≥ 7.1 g (11.5%).

There was a perfect agreement between the results of DAT and microscopic cyst detection in mice (Kappa=0.85, P<0.0001).

Discussion

In the present study, the overall seroprevalence recorded in pigs was found to be 38.28%, which is in close agreement with the prevalence estimated in previous studies in China [20], Brazil [21] and Zimbabwe [22]. Lower prevalence values of 0.4%, 2.1%, 14.4 %, and 16.1% were recorded by Deksne and Kirjusina [23] in Latvia, Iovu et al. [24] in Romania, Huang et al. [25] in China and Veronesi et al. [26] in Italy, respectively. Much higher prevalence (95.8%) was reported by Ortega-Pacheco et al. [27] in Mexico. Nevertheless, close comparisons of seroprevalence between the present study and the aforementioned studies might be difficult, because of the difference in the management practices of pigs, sample size, the relative cat densities, climate, the diagnostic techniques and the cut-off values used [3,28,29]. In the present study, the use of DAT, the most sensitive and specific diagnostic test recommended for the screening of sera in several animals and humans [2], coupled with the extensive management of pigs might have enabled us to get significantly high seroprevalence and isolation rate in mice.

The observed isolation rate of *T. gondii* from seropositive pigs (24/50, 48%) is in close agreement to 4 of 8 (50%) [30], and 20 of 40 (50%) [31], seropositive pigs from Brazil. It is lower compared with 39 of 69 (56%) bioassayed pigs from USA [32] and 16 of 22 (73%) seropositive pigs from Serbia [33]. It is higher compared to 3 of 22 (13.63%) [34], and 50 of 170 (29.41%) [35], seropositive pigs from USA, 7 of 28 (25%) seropositive pigs from Brazil [36] and 7 of 28 (25%) seropositive pigs from Portugal [37]. These differences may be due to the density of *T. gondii* in tissues of pigs and the type of tissues used for bioassay. *Toxoplasma gondii* localizes more often in muscle than the brain of pigs [2]. In addition, these findings are probably due to the type of bioassay used (cats versus mice) and the number of mice used (10 mice versus 5 mice) and the amount of tissue sampled (50 g versus 100 g) [38].

One of the twenty-six *T. gondii* isolates which originated from a pig from the Bishoftu area was mouse virulent (killed 4 of 5 infected mice between 28-29 days of post inoculation). Previous studies in central Ethiopia indicated that 9.1% (3/33) of the *T. gondii* isolates in sheep and goats [39] and none of the 24 *T. gondii* isolates in free range chickens [40] were virulent for mice. The present result is in contrast with the findings of Pena et al. [16], who reported a high percentage of mice pathogenic isolates from Brazil. The majority of the isolates in the present study developed chronic infection in mice. This might be due to the genotype of *T. gondii* strains or smaller doses of the parasite in the homogenate inoculated or release of a small number of bradyzoites from tissue cyst during pepsin digestion [41].

Significant statistical association was found between the titres of the tested pigs and isolation in mice. Demonstration of a high percentage of mice (34.4 %) with viable parasites from samples of pigs with high antibody titer (>6000) indicates that the seropositive pigs with high antibody titer quite frequently harbor live parasite in their tissue. Similar results were also observed previously in chickens and cats [16,42].

High percentage of mice (25.8%) with mean weight difference <7.1 g (didn't gain weight much) are more likely to harbor cysts in their brain than those mice who gained weight more than the mean weight difference of >7.1 g (11.5 %) (P=0.008) suggesting possible effect of *T. gondii* infection on weight gain of mice.

Viable *T. gondii* was also isolated from two pooled pig tissue samples with DAT negative results. Similar results were reported by Omata et al. [43] who isolated *T. gondii* from 2 of 40 indirect fluorescent antibody tests (IFAT) negative pigs (1:16). Hejliiek and Literik [44] also isolated *T. gon*dii from 18 of 38 dye test-negative pigs. The isolation of *T. gondii* from seronegative pigs indicates that either these pigs were recently infected and had not yet developed *T. gondii* antibodies, or that the antibody titers had declined to undetectable levels. The latter is more likely, because most pigs in our study were adults and most likely they had been infected for several months.

Epidemiological and population studies with multilocus PCR-RFLP or microsatellite markers have shown that *T. gondii* isolates from South America are highly diverse and distinct from those from North America and Europe, where Type II is predominant [45,46]. The few studies carried out on genotyping of *T. gondii* isolates from 27 feral cats from Addis Ababa, Ethiopia [47] and 33 isolates from sheep and goats of central Ethiopia [39] indicated limited genetic diversity with dominance of Type II and few cases of Type III and atypical genotypes.

Conclusions

The results of the present study confirm the widespread presence of *Toxoplasma gondii* viable tissue cysts in pigs in Ethiopia. The results suggest that large amounts of pork containing viable *T. gondii* are released to markets for consumers. Therefore, enforcing hygienic measures, public education, and provision of rendering facilities to kill the bradyzoites in meat whenever possible and further studies deserve consideration. This is the first report of isolation of viable *T. gondii* in pigs in Ethiopia.

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