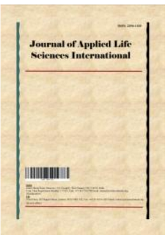

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Isolation and Cryopreservation of *Toxoplasma gondii* Isolates from Cats and Chickens from Selected Households in the Thika Region, Kenya

Adele Njuguna [a], Naomi Maina [b], John Kagira [c*], Simon Karanja [d], David Kamau [d], Maina Ngotho [e], John Mose [f] and Lucy Mutharia [b]

[a] *Department of Natural Sciences, Catholic University of Eastern Africa, P.O. Box 62157-00200, Nairobi, Kenya.*
[b] *Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya.*
[c] *Department of Animal Sciences, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya.*
[d] *Department of Public Health, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya.*
[e] *Department of Clinical Studies, University of Nairobi, P.O. Box 29053, Nairobi, Kenya.*
[f] *Department of Biomedical Science and Technology, The Technical University of Kenya, P.O. Box 52428, Nairobi, Kenya.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

There is a shortage of information in Africa regarding the genotypic and phenotypic characteristics of the *Toxoplasma gondii* circulating in domestic cats (*Felis catus*) and the intermediate hosts such as chicken. The situation is compounded by a lack of collection of well-stored isolates. The present study was aimed at creating a cryobank of *T. gondii* bradyzoites, tachyzoites, and oocysts. The parasites were isolated from cats and chickens kept in households in the Thika region, Kenya. Eight (8) cat fecal samples positive for *T. gondii* oocysts and 38 chicken brain tissue cysts (bradyzoites) were obtained and used for propagation in mice before cryopreservation. For each

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*Corresponding author: E-mail: jkagira@gmail.com, jkagira@jkuat.ac.ke;

sample from the cats, (two donors) BALB/c mice were infected orally with 1 x 10^4/ml of oocysts. From each chicken sample, (two donors) BALB/c mice were infected intraperitoneally with 20-30 tissue cysts. On the third day after infection, tachyzoites were harvested from the peritoneal cavity of one donor mouse. The other two infected mice were further monitored for eight weeks, euthanized and the brain tissue harvested for toxoplasma cysts which were purified and cryopreserved. From the mice infected with oocysts from the cats' samples, 2 (25%) tachyzoites but a higher 8 (100%) isolation was obtained from brain tissue cysts. On the other hand, from chicken samples generated 18 (47.3%) tachyzoites and 38 (100%), tissue cysts were obtained. The isolated oocysts (from cats), tachyzoites, and tissue cysts (from mice) were cryopreserved using 15% glycerol as cryoprotectant and stored in liquid nitrogen (-196^{o}C). After 6 months of cryopreservation, the viability of the isolates was tested using Trypan blue dye exclusion on a manual hemocytometer. Viability (99.5% - 96%) of the cryopreserved samples was maintained for the three toxoplasma stages and there was no significant change ($p>0.05$) in the viability of the parasites before and after cryopreservation. The cryobank will serve as a repository for subsequent studies on molecular and phenotypic characterization of *T. gondii* isolates from Kenya.

Keywords: Chicken; cat; cryopreservation; tissue cyst; oocyst; tachyzoites; Thika.

1. INTRODUCTION

Toxoplasma gondii, which causes toxoplasmosis, infects all warm-blooded animals, with the felids family being the definitive host while other animals are intermediate hosts. The parasite is mainly transmitted by fecal-oral, carnivorism, and trans-placental routes. The prevalence of toxoplasmosis in human populations varies according to countries, geographical areas, and ethnic groups [1]. A recent review also showed that in most African countries, there is a high-risk area mainly due to the close association of humans and livestock as well as socio-cultural practices, and poor environmental hygiene [2]. In Kenya, the seroprevalence of up to 60% has been reported in epidemiological in different categories of people [3,4,5]. Ongoing studies by the current investigators show that in Kenya, just like in other countries, the risk factors associated with toxoplasmosis include the keeping of free-ranging domestic cats and chickens, which harbor *T. gondii* oocysts and tissue cysts [6,7,8].

The phenotypic and genotypic characteristics of *T. gondii* are critical in determination of pathogenesis, virulence, diagnosis and treatment of the ensuing disease [9]. Felids excrete oocysts which sporulate in the environment. The sporozoites are ingested by intermediate hosts through various modes (drinking water, eating raw vegetables). Human may also get infected by consuming raw or undercooked meat containing tissue cysts stages [10]. The tachyzoites in the intermediate hosts are associated with the acute phase of infection while tissue cyst is the encysted form and contains bradyzoites. The latter is also the terminal stage in intermediate hosts and infective to felids.

Most of the information regarding *T. gondii* phenotypic and genotypic characteristics emanates from studies done in Europe and America [11], with little being documented from the African region. Isolation of viable *T. gondii* in the laboratory is usually maintained by propagation in mice via serial passages. It faces critical challenges on cost, ethical issues based on laboratory animal use, and labor intensity [12]. Cryopreservation of isolates provides an alternative to laboratory animals [13]. The aim of this study was to create a cryobank of all infective stages of *T. gondii* isolated in Kenya for further studies.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out in the Thika region; (latitude 1° 4' 60 S, longitude 37° 4' 60 E) located in Central Kenya, Kiambu County, and covering an area of approximately 1,960Km². The region has a tropical climate with an annual rainfall ranging between 500 mm – 1500 mm and an annual average temperature range of 16.4 to 19.8^{o}C. The main economic activity in the area is livestock agriculture. Most homes in this environment keep domestic cats as pets and curiously/traditionally for controlling rodents [6]. Samples from cats and chickens in selected households in a peri-urban setting were collected from Ruiru, Juja, and Thika sub-counties.

2.2 Laboratory Animals

BALB/c mice were obtained from Institute of Primate and Research (IPR) -Nairobi, breeding colony. The mice were housed in standard shoe box macron cages in groups of five per cage in a BSL2 laboratory at IPR. They were provided with beddings and other environment enrichment items such as tunnels for burrowing. The mice were fed with mice pellets (Mice pellets®, Unga Ltd, Kenya) and provided drinking water *ad libitum* as detailed by IPR guidelines. The ambient room environment temperature was maintained at between 22 and 27^0C.

2.3 Isolation of Oocysts and Tissue Cysts from Cats and Chicken

The oocysts were obtained and processed from fecal samples of eight (8) cats as described by [14]. In brief, the oocysts were isolated by the sugar flotation technique [15] and observed at a magnification of x400. The oocysts were confirmed to be *T. gondii* using a polymerase chain reaction (PCR) targeting 529 bp [14,16].

For tissue cysts isolation, 48 free-range chickens were purchased from local farmers and sacrificed by a registered veterinary Laboratory Technician using the cervical dislocation method as described by [17]. The tissue cysts were isolated from 38 (79%, 38/48) of the chicken brain tissue. Briefly, the chickens were sacrificed (within 48 hours) and their brains removed as previously described by [18]. The yield on isolated cysts was enumerated using the manual counting method in a hemocytometer [14].

2.4 Propagation of *T. gondii* in Mice

Sporulated oocysts (10^4 in 0.2 ml) from each fecal sample were administered (for expansion/multiplication) by oral gavage into two sets of BALB/c mice per sample. After three days of inoculation, the first group of mice was sacrificed using standard CO_2 euthanasia. The tachyzoites were harvested from the peritoneal cavity of the mouse using the method described by [19]. The numbers of tachyzoites were determined by manual counting on a hemocytometer. The second group of inoculated mice was monitored for eight weeks after which tissue cysts from the brain were harvested.

The process was repeated for cysts obtained from the chicken brain. In this case, the cysts were diluted using phosphate saline glucose (PSG) into 20-30 cysts per 0.2 ml. The latter dose was injected intraperitoneally into two sets of BALB/c per sample of oocysts from the chicken and harvested as described by [20].

2.5 Cryopreservation of Isolated Parasites

A 500µl suspension of oocysts, tachyzoites, and tissue cysts containing bradyzoites were mixed with an equal amount of 500µl 15% glycerol. Aliquots of 200µl for tachyzoites and oocysts and 500 µl for tissue cysts were cryopreserved. The aliquots were cooled in a stepwise manner at -20^0C for 4hrs, -80^0C overnight, and then transferred into liquid nitrogen (-196^oC) as described by [21].

2.6 Viability Testing

The cryopreserved tachyzoites, tissue cysts, and oocysts were retrieved from liquid nitrogen after 6 months of storage. Following rapid thawing at 37^oC in a water bath, samples were then diluted 1:5 in RPMI with 5% FCS, centrifuged, and suspended in PBS for viability assessment. Further, the samples were diluted in sterile-filtered Trypan Blue dye by preparing a 1:1 dilution of the cell suspension using 0.4% Trypan Blue solution. 20µl was loaded on a hemocytometer and observed under x 40 eyepiece of light microscope live/dead parasites quantified. The motility of tachyzoites was also used as a confirmatory test to assess their viability.

2.7 Data Analysis

Data was managed and analyzed using Ms Excel® software (Microsoft, USA). Paired ANOVA test was used to correlate viability of *T. gondii* oocysts, tissue cysts and tachyzoites, before and after cryopreservation.

3. RESULTS

3.1 Proportion of Tachyzoites and Tissue Cysts Harvested from Mice

Toxoplasma gondii tachyzoites were isolated from 25% (2/8) of cat fecal samples. Further, all (100%) mice infected with oocysts survived for eight (8) weeks after infection. The mice remained asymptomatic throughout the eight weeks of monitoring. However, brain tissue cysts containing bradyzoites were obtained in all eight (8) study mice.

Following infection of the 38 *T. gondii* from chicken tissue samples into BALB/C mice, 18 (47.3%) tachyzoites. The mice remained asymptomatic throughout the eight weeks of monitoring but all 38 (100%) samples from chicken brain cysts led to brain tissue cysts in the infected mice.

3.2 Viability of Cryopreserved *T. gondii* Isolates

After the cryopreservation for 6 months of the three stages of the parasites, the viability of the isolates was done and the results are shown in Table 1.

The cryopreserved parasites (oocysts, bradyzoites, and tachyzoites) were not morphologically distinguishable from the fresh ones. The percentage of surviving parasites was similar and high ranging from 96 to 99.5%. The viability was highest for the oocysts (99.5%). There was no significant ($p>0.05$) difference in the viability of the parasites before and after cryopreservation (Table 1).

4. DISCUSSION

Toxoplasmosis is a neglected disease affecting both animals and public health with high risk of morbidity in developing countries [2]. In Africa, limitations in addressing gaps in the management of toxoplasmosis study are hindered by a lack of repertoire of preserved isolates. This study was geared towards the isolation, and propagation of isolates collected from cats and chicken hosts from an area known to be endemic to the disease [5,7].

In the present study, *T. gondii* oocysts from cats were able to generate tachyzoites and brain tissue cysts. However, although all mice injected with oocysts developed the brain tissue cysts, the harvested tachyzoites from the peritoneal cavity were quite low. Similarly, the tissue cysts obtained from the chicken also generated fewer tachyzoites than brain tissue cysts in the mice. This could be due to the low sensitivity of the peritoneal harvesting method in obtaining all the tachyzoites from the mice. Previous studies have shown tachyzoite formation in the peritoneal cavity ranges from a few hours to four days with non-virulent strains taking a longer period than virulent ones [22]. Studies done on *T. gondii* cultivation indicate variation in tachyzoite formation depends largely on the genotype of the isolate; the strain of mice used and route of infection are crucial factors to the outcome of infection [15,23]. In the current study, oocysts generated fewer (25%) tachyzoites compared to tissue cysts from chicken which generated 47.3% tachyzoites in the mice. This could be due to differences in the host source, the genotype of the parasite, and the route of infection. Further studies should be undertaken to determine the genotypes of these isolates.

Once the parasites multiply in epithelial cells of the small intestine and after that in the peritoneal cavity, *T. gondii* has a tropism for other cells, with the brain being the main target. Thus, in the current study, all mice infected either by oocysts or tissue cysts from chicken developed brain tissue cysts that had bradyzoites. This study is similar to previous studies showing that *T. gondii* primarily targets brain cells [24,25].

Cryopreservation, which was used in this study, has the advantage of maintaining the strains in their original state and not being modified as is common during continuous laboratory animal passage. The latter is costly and laborious [13]. The current study showed that most of the oocysts, tachyzoites, and tissue cysts containing bradyzoites were able to maintain their morphology after cryopreservation. This shows that cryopreservation did not significantly affect the integrity of the parasites and could thus produce experimental infections. Higher cryopreservation success (over 90%) was noted in this study. Thus, cryopreservation which was done using 15% glycerol, can be used during the long-term storage of all the three stages of *T. gondii* parasites. Previous studies have shown that glycerol can be used as a cryoprotectant for other cells such as spermatozoa, other mammalian cells, and parasites including *Entamoeba histolytica*, *Trichomonas vaginalis*, *Leishmania* spp., *Trypanosoma* spp. [21,26,27]. Cryopreservation creates a dehydrating environment during freezing, thus preserving the cells from cryo-injuries. Glycerol was used in this study, and it is known to form hydrogen bonds with water molecules, thus preventing ice crystals formation, which damages cells [28].

Availability of repertoire of isolates of viable *T. gondii* infective stages from developing countries like Kenya will provide a platform to study the relationship between the genotypic and phenotypic characteristics of local isolates of *T. gondii.* Most of the information available on *T. gondii* is from Europe, Asia, and America, with little documentation from African countries. The

Table 1. Viability of *Toxoplasma gondii* after six months cryopreservation

Parasite Stage	Original host	Mean No. (Parasites/ml) at storage	Mean No. (Parasites/ml) after 6 months cryopreservation	Viability (%) after 6 months cryopreservation
Oocysts	Cat	$7.25x10^2$	$7.21x10^2$	99.5
Tachyzoites	Cat	$2.49x10^7$	$2.49x10^7$	97
Tissue cysts	Cat	$1.60x10^4$	$1.60x10^4$	96
Tissue cysts	Chicken	$1.05x10^7$	$1.05x10^7$	97

cryopreservation method of storage of parasites is able to save laboratory workers a considerable amount of the time, effort, and expense involved in the frequent serial passage of the parasite in laboratory rodents. Maintenance of *T. gondii* in laboratory animals has been associated with genome changes characterized by loss of ability to form tachyzoites, gametocyte-forming ability, and the selection of some mutations by the host animal or growth system [29].

5. CONCLUSION

This study has been able to isolate, and cryopreserve *T. gondii* isolates from cats and chickens in Kenya. The genotypic and phenotypic characterization of these isolates will ensue with the intention of determining their relatedness with isolates from other continents and their impact on disease pathogenesis and management. There is a need for an integrative, multidisciplinary approach to control toxoplasmosis, a disease of public health significance.

DISCLAIMER

The consumables used for this research are commonly used in our area of research. There is absolutely no conflict of interest between the authors and producers of the consumables and we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

ETHICAL CLEARANCE

Ethical clearance for the studies involving animals was approved by Institutional Animal Care and Use Committee of the Institute of Primate Research, Nairobi (Ethical clearance certificate IRC/21/11). The study also adhered to the ARRIVE guidelines for reporting in vivo animal experiments.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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