Genotyping of Toxoplasma gondii from Stray Cats by Multilocus PCR-RFLP Techniques in Erbil city -Kurdistan Region/Iraq.

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Abstract. In this study, ten multilocus PCR-RFLP markers were used for genotyping of T. gondii in six cats that were seropositive by MAT test. These markers include SAG1, SAG2 (5-SAG2, 3-SAG2 and Alt.SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico. Eight T. gondii reference strains (GT1, PTG, CTG, TgCgCa1, MAS,TgCatBr5, TgCatBr64 and TgToucan) were used as positive controls for genotyping. Sample A20-1 was type III by one marker (GRA6), while sample A9-2 was type III by four markers (SAG3, BTUB, c22-8 and c29-2) and sample A20-2 was type III by four markers (SAG1, SAG3, BTUB and c29-2). Hence, it could be concluded that the strain of T. gondii in Kurdistan stray cats is likely the type III.

Keyword: Toxoplasma gondii; Multilocus PCR-RFLP genotyping, Stray cats

1-Introduction:
Toxoplasma gondii is one of the most successful coccidian protozoans with potential zoonotic impact among humans, other mammals and birds worldwide. It is capable of infecting virtually all warm-blooded animals, infecting both mammals and birds (Tenter et al., 2000 and Dubey, 2010).

The life cycle of the parasite includes an asexual reproduction in the intermediate hosts (mammals and birds) and sexual reproduction in the definitive host (feline). Ingestion of sporulated oocysts by drinking water or eating unwashed vegetables, eating raw or undercooked meat containing tissue cysts are the main transmission routes in humans and animals (Carme et al., 2002 and Heukelbach et al., 2007)

Cat’s act as definitive hosts for the T. gondii and they play a significant role in spreading the oocysts in the environment (Dubey et al., 1998 and Tenter et al., 2000). Seroprevalence studies in cats indicate that T. gondii infection increases with age and are more common in feral cats than in domestic cats (Dubey and Beattie, 1988).
The distribution of *T. gondii* genotypes varies in different geographical regions (Lehmann *et al.*, 2006). Initially, *T. gondii* strains were classified into three clonal lineages, namely types I, II, and III (Howe and Sibley, 1995). Conventional designation defined the clonal Type I, II, and III lineages, and the others are lumped together as atypical or exotic genotypes (Howe and Sibley, 1995; Grigg *et al.*, 2001 and Su *et al.*, 2006). The virulence of the type is linked to its infectivity to outbred mice, type I isolates are 100% lethal to mice, irrespective of the dose, and that types II and III are less virulent for mice (Sibley and Boothroyd, 1992 and Howe *et al.*, 1996). Identification of *T. gondii* strains is often achieved by Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) (Howe and Sibley, 1995 and Su *et al.*, 2006 and 2010). Since no attempt has been made so far to study the genotyping of *T. gondii* isolated from cats using different genetic markers and cell culture of *T. gondii*, therefore this study is the first study in Erbil as well as in other parts of Iraq in this direction.

2. Material and Methods

2.1 Sample collection

One hundred stray cats were hunted by trap cage and shooting tranquilizer gun in Erbil city from June 2015 to March 2016 and used for detecting *T. gondii* antibodies by MAT test, then genotyping. For temporary anesthetizing the cats, blowpipe syringe tranquilizer (B11) which contained 0.6 mL (100mg/mL) of ketamine chlorhydrate and 0.4 mL (8 mg/mL) of xylazine was used. The cats were transferred to animal house, reared for 30 days, with good biosecurity plan. Before withdrawing the blood, the animals were sedated by I.M injection with Medetomidine (0.4 mL). Three mL of the blood was taken from the cephalic vein using a sterile disposable syringe; the blood was transferred to labeled tubes without anticoagulant. Each tube was labeled clearly and transferred to the immunological lab of biology department – Collage of science at Salahaddin University for MAT test.

2.2 MAT Test

The MAT test (KeraFast, EH2001, USA) was performed in Biology department college of science, Salahaddin University following the procedure described by Desmonts and Remington (1980) and Dubey and Desmonts (1987). The MAT test was carried out using 96 well U-bottom microtiter plates. Serum samples were serial diluted from 1:25 to 1:3200. Positive and negative controls were included for each microtiter plate. The cutoff for MAT titer is 1:25, titers >=1:25 is considered seropositive for *T. gondii* infection.

2.3 Heart Tissue Samples

The heart tissue of 12 seropositive cats with the MAT titers of 1:2400-1:800 to *T. gondii* following the following steps:

1. The cats were anesthetized by using 0.6 mL (12 mg/ml) of ketamine chloride and 0.4 mL of Xylazine as a general anesthesia.
2. Five mL lidocaine was injected directly between 5th and 6th chest ribs to the heart for the euthanasia.
3. A longitudinal incision about 15 cm. was made from the chest to the abdomen passing through the muscle layers, peritoneum, and diaphragm. The thoracic cavity was opened and the heart was take under sterile condition.
4. The isolated cat heart tissues were kept in zip-lock transparent plastic bag inside an icebox, then shipped by DHL to Dr. Su lab, at the Microbiology Department / University of Tennessee, Knoxville, USA.

The samples were arrived within three days, these samples were used for Multilocus PCR-RFLP genotyping of *T. gondii*.

2.4 PCR-RFLP Technique

DNA was extracted from *T. gondii* using DNeasy blood and tissue, Kit (Qiagen, USA, 2006), the procedure was performed according to the instructions provided with the kit. While for Multilocus PCR-RFLP genotyping, Multiplex PCR, Nested PCR and Restriction enzyme for three successive
steps was used. The three procedures were performed according to the protocol of Dr. Su’s laboratory (Su et al., 2010) Department of Microbiology, University of Tennessee, Knoxville, USA.

**Results:**

The serum samples of 100 cats were tested by MAT test. From each positive cat two samples with titer equal or greater than 1:50 were selected for genotyping. The six strong seropositive cats were dissected (Table 1); the heart form each cat was taken for DNA extraction, amplification. Ten multilocus markers for PCR-RFLP genotyping were used, which included SAG1, SAG2 (5'-SAG2, 3'-SAG2 and Alt.SAG2) SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico with different restriction enzymes (Table 2). From positive samples, three samples (A20-1, A9-2, and A20-2) gave DNA band which indicated as type III (Table 3 and Figure 2) by comparing with control positives which include GT1, PTG, CTG, TgGcGa1, MAS, TgCatBr5, TgCatBr64 and TgToucan (Table 4 and Figure 1).

### Table 1: Seropositive samples used of T. gondii IgG Abs by MAT for genotyping of cat heart tissue.

| Samples | MAT titer |
|---------|-----------|
| A9-1    | 1:400     |
| A10-1   | 1:200     |
| A18-1   | 1:800     |
| A19-1   | 1:400     |
| A20-1   | 1:400     |
| A21-1   | 1:400     |
| A9-2    | 1:400     |
| A10-2   | 1:200     |
| A18-2   | 1:800     |
| A19-2   | 1:400     |
| A20-2   | 1:400     |
| A21-2   | 1:400     |

### Table 2: Genetic marker with restriction enzymes for multi-locus PCR-RFLP genotyping.

| Genetic loci Markers | Restriction enzymes | References |
|----------------------|---------------------|------------|
| SAG1                 | Sau96I + HaeII (double digest) | Grigg et al., 2001 |
| 5-SAG2               | MboI                | Su et al., 2006; Howe et al., 1997 |
| 3-SAG2               | HhaI                | Howe et al., 1997 |
| SAG3                 | NciI                | Grigg et al., 2001 |
| BTUB                 | BsiEI + TaqI (double digest) | Su et al., 2006 |
| GRA6                 | MseI                | Fazaeli et al., 2000; Su et al., 2006 |
| c22-8                | BsmAI + MboII (double digest) | Su et al., 2006 |
| c29-2                | HpyCH4IV + Rsal (double digest) | Su et al., 2006 |
| L358                 | HaeIII + NalIII (double digest) | Su et al., 2006 |
| PK1                  | Aval + Rsal (double digest) | Su et al., 2006 |
| Alt.SAG2             | HinfI + TaqI (double digest) | Su et al., 2006 |
| Apico                | AflII + DdeI (double digest) | Su et al., 2006 |
Table (3): Multilocus PCR-RFLP genotyping results by 10 genetic markers (cat heart tissue)

| Strains  | SAG1 | SAG2 | SA G3 | BTUB | GRA6 | C22-8 | C29-2 | L358 | PK1 | Alt. | Apico |
|---------|------|------|-------|------|------|-------|-------|------|-----|------|-------|
| A9-1    | NB²  | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A10-1   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A18-1   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A19-1   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A20-1   | NB   | NB   | NB    | III² | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A21-1   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A9-2    | NB   | NB   | III   | III   | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A10-2   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A18-2   | NB   | NB   | III   | III   | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A19-2   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A20-2   | III  | III  | III   | III   | NB   | NB    | NB    | NB   | NB  | NB   | NB    |
| A21-2   | NB   | NB   | NB    | NB    | NB   | NB    | NB    | NB   | NB  | NB   | NB    |

1. NB= no band  2. Type III allele

Table (4): Eight references strain used as positive control for genotyping (Su et al., 2010)

| Genetic Markers | Strains | Host | Local. | SA G1 | (5'+3')SA G2 | Alt.SA G2 | SA G3 | BTU B | GRA6 | C22-8 | C29-2 | L358 | PK1 | Apico | Genotype |
|-----------------|---------|------|--------|-------|--------------|-----------|-------|-------|------|-------|-------|------|-----|-------|----------|
| GT1             | Goat    | USA  | I      | I     | I             | I         | I     | I     | I    | I     | I     | I    | I   | I     | I        |
| PTG             |         | USA  | II or III | II   | II            | II        | II    | II    | II   | II    | II    | II   | II  | II    | II       |
| CTG             | Cat     | USA  | II or III | II   | II            | II        | II    | III   | III  | III   | III   | III  | III | III   | III      |
| TgCgCa1 MAS     | Cougar  | Canada | I      | II    | II            | II        | III   | III   | III  | u-1   | I     | I    | u-1| I     | Atypical |
| MAS             |         | Human | u-1    | I     | II            | III       | III   | u-1   | I    | I     | III   | I    | III | I     | Atypical |
| TgCatBr5       | Cat     | Brazil | I      | I     | III           | III       | III   | III   | I    | I     | u-1   | I    | I   | I     | Atypical |
| TgCatBr64      | Cat     | Brazil | u-1    | I     | III           | III       | III   | u-1   | I    | I     | III   | I    | I   | I     | Atypical |
| TgToucan       | Toucan  | Costa Rica | u-1   | I     | II            | I         | III   | u-2   | I    | I     | I     | I    | I   | I     | Atypical |
| (TgRsCr1)      |         |       |        |       |               |           |       |       |      |       |       |      |     |       |          |
Figure (1) Multiplex Multilocus nested PCR-RFLP (Mn-PCR-RFLP) analysis of *T. gondii* samples using 10 different genetic markers with the *T. gondii* reference strains: GT1 (or RH88), PTG, CTG, TgCgCa1 (a.k.a. Cougar, COUG), MAS, TgCatBr5, TgCatBr64 and TgRsCr1. Nested PCR products from each marker were digested with selected restriction enzymes and DNA fragments were separated in agarose. All markers were resolved in 2.5% gel, except that Apico was resolved in 3% gel. MK, low molecular weight DNA ladder (New England Bio labs Inc).
Figure (2) Multiplex Multilocus nested PCR-RFLP (Mn-PCR-RFLP) analysis of *T. gondii* samples from Cats using 10 different genetic markers. Six of genetic markers were positive with clear bands by comparing with positive controls for detecting *T. gondii* genotypes. The six genetic markers are:

1- Type III by *SAG1* loci genetic marker.
2- Type III by *SAG3* loci genetic marker
3- Type III by *BTUB* loci genetic marker
4- Type III by *c22-8* loci genetic marker
5- Type III by *GRA6* loci genetic marker
6- Type III by *c29-2* loci genetic marker.
Discussion:

The 10 Multilocus markers, SAG1, SAG2 (5'—SAG2, 3'—SAG2 and Alt.SAG2) SAG3, BTUB, GRA6, c22-8, c29-2, L358 and PK1 used for PCR-RFLP genotyping in the present study, were used widely in identifying *T. gondii* isolates from a variety of hosts in different geographical regions (Su *et al*., 2006; and Pena *et al*., 2008).

In this study the sample A20-1 was type III (typical) by one locus Marker GRA6, the coding region of GRA6 locus is a considerable polymorphic target and can clearly differentiate the three archetypal genotypes (I, II and III) by using a single endonuclease digestion with MseI (Fazaeli *et al*., 2000). Regarding sample A20-2 it was type III by four locus markers SAG1, SAG3, BTUB and c29-2, respectively and the sample A9-2 was also type III by SAG3, BTUB, c22-8 and c29-2, respectively. The SAG1 marker is based on the digestion of the PCR product by the enzymes Sau 96I and HaeII (Sibley and Boothroyd, 1992). This double digestion of SAG1 identified sample A20-2 as Type III while, the Marker SAG3 required only NciI enzyme to discriminate the three archetypal alleles during one digestion reaction (Grigg *et al*., 2001) and they gave pattern as type III in both sample (A20-2 and A9-2). Regarding BTUB Marker, a double digestion with BsiEI and TaqI were used to distinguish the three archetypal alleles (Su *et al*., 2006), and characterized both sample (A20-2 and A9-2) as type III, while Marker c29-2 with HpyCH4IV and RsaI which are also with double digestion, characterized the samples A20-2 and A9-20 as type III. Also Marker c22-8 required double digest which are BsmAI and MboII (Su *et al*., 2006) and characterized the sample A9-2 as type III.

From the current study it can be concluded that *T. gondii* isolated from cat heart tissue by six genetic markers (SAG1, GRA6, SAG3, BTUB, c22-8 and c29-2) was type III. The type III is also name as ToxoDB genotype #2 (Shwab *et al*., 2014). The results of this study are in agreement with the results of Tavalla *et al*., (2013) in Iran who identified type III from 9 positive sample out of 150 soil samples containing toxoplasma oocysts collected from garbage dumps, children's schoolyard, parks and free places. Also, this study is in agreement with the study of Al-Kappany *et al*., (2010) in Egypt who identified 42 samples of type III among 115 tissue samples of feral cats using SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico markers.

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