Molecular detection of *Toxoplasma gondii* in house sparrow (*Passer domesticus*) by LAMP and PCR methods in Tehran, Iran

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Abstract *Toxoplasma gondii* is one of the most common zoonotic parasitic diseases in human and warm-blooded animals worldwide. Birds are one of important intermediate hosts of *T. gondii*. The aim of this study is molecular detection of *T. gondii* in the house sparrow by LAMP and PCR methods in Tehran, Iran. A total 200 sparrows were captured in different regions of Tehran. DNA was extracted from tissue samples of each sparrow. LAMP and conventional PCR assays were carried out with a set of primers to detect the 529 bp fragment of *T. gondii*. LAMP and PCR were detected *T. gondii* from 17 (8.5 %) and 15 (7.5 %) of 200 sparrows respectively. These results indicated that sensitivity of LAMP was higher than conventional PCR. In our knowledge, this study is the first report of detection of *T. gondii* by LAMP method in bird hosts. Also, these findings provided an insight into epidemiological pattern of *T. gondii* infection in sparrow in Iran.

Keywords *Toxoplasma gondii* · Sparrow · Loop-mediated isothermal amplification (LAMP) · PCR · Iran

Introduction

Toxoplasma gondii is a widely prevalent protozoan parasite in human and warm-blooded animals worldwide (Tenter et al. 2000). Humans usually get infected through consuming drink or food contaminated with oocysts or by ingesting tissue cysts from undercooked meat. Felids, including domestic cats are definitive hosts in the life cycle of *T. gondii*. Cats become infected through eating infected tissues of intermediate hosts (Tenter et al. 2000). *T. gondii* infected a wide variety of warm-blooded animals including birds (Dubey 2002, 2010). Birds are one of important intermediate hosts of *T. gondii*, because infection in birds is one of the best indicators for environmental contamination with *T. gondii* oocysts due to their dietary habit. Moreover, infected birds are considered a good source of infection for human and cats (Dubey 2010). The house sparrow (*Passer domesticus*) is one of the common birds that easily adaptable to urban and rural areas. Recently, high molecular frequency of *T. gondii* infection in sparrow were reported from Iran (Khademvatan et al. 2013), China (Cong et al. 2013; Huang et al. 2012) and Brazil (Gondim et al. 2010; Vilela et al. 2011).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method technology that amplifies DNA under isothermal condition with high sensitivity, specificity, and rapidity (Notomi et al. 2000). LAMP has been developed for molecular detection of several microorganisms [reviewed in (Mori et al. 2013)], including *T. gondii* (Kong et al. 2012; Lin et al. 2012; Zhang et al. 2009).

There is little information about molecular frequency of *T. gondii* in bird hosts in Iran (Asgari et al. 2009; Khademvatan et al. 2013; Zia-Ali et al. 2005). Hence, the aim of this study is determination of molecular prevalence

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of *T. gondii* in the house sparrow by LAMP and PCR methods in Tehran, center of Iran.

Materials and methods

Study area

Tehran province is located to the north of the central plateau of Iran. Tehran covers an area of about 12,981 square kilometres, and is located at 34–36.5° longitude north and 50–53° longitude east. Tehran has over 12 million inhabitants (17.5 % of Iran population) (2014). Tehran city has hot summers and moderate winters (ranging between 28 and 30 °C from mid-July to mid-September and around 1 °C from December to January) with an average annual rainfall of 200 mm (Farhadian 2011).

Sample collection and DNA extraction

A total 200 sparrows were captured (using traps) in different regions of Tehran from February to November 2013. Brain samples of each sparrow were homogenized and used for DNA extraction. Briefly, 50–70 mg of homogenized samples diluted with 800 µL of lysis buffer (50 mM Tris–HCl, pH8.0; 25 mM EDTA and 400 mM NaCl), 100 µL 10 % SDS (Biase et al. 2002), and 30 µL Proteinase K (20 µg/µL) (Fermentas, USA). After incubation at 55 °C for 3 h, proteins and undissolved debris were precipitated by adding 300 µL 6M NaCl, incubation at 4 °C for 15 min (Biase et al. 2002). Then, the samples were extracted with phenol–chloroform–isoamyl alcohol (25:24:1). DNA was precipitated by adding twice volume of 100 % ethanol and sodium acetate solution (3 M, pH 5.2), kept at –70 °C for 60 min, followed by centrifugation at 13,000×g for 5 min. Then the pellet washed twice with 70 % ethanol and resuspended in 100 µL of double-distilled water and stored at –20 °C until use.

Conventional PCR

Conventional PCR was performed using a pair of *T. gondii*-specific primers TOX4 (5'-CGCTGCAGGGAGGAA-GACGAAAGTTG-3') and TOX5 (5'-CGCTGCAG ACA-CAGTGCATCTGGATT-3') as previously described by Homan et al. (2000), amplifying a region of 529 base pairs (bp) fragment and repeated 200–300 times in *T. gondii* genome (Homan et al. 2000). Amplification was performed with initial denaturation for 7 min at 94 °C, followed by 30 cycles for 35 s of 94 °C, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 10 min.

LAMP reaction

The LAMP assay was performed according to an earlier report with a set of four specific primers to detect the 200- to 300-fold repetitive 529 bp fragment of *T. gondii* (Kong et al. 2012; Lin et al. 2012). All samples were carried out in 25 µL reaction mixtures containing 40 pmol (each) of primers FIP and BIP, 5 pmol of primers B3 and F3, 1.4 mM of deoxynucleoside triphosphates (dNTP), 8 U of *Bst* DNA polymerase (New England Biolabs, USA), 2× reaction buffer (1.6 M betaine (Sigma-Aldrich), 40 mM of Tris–HCl (pH 8.8), 20 mM of KCl, 20 mM of (NH₄)₂SO₄, 16 mM of MgSO₄, and 0.2 % Tween 20) and 1 µL of template DNA. To determine the optimal time and temperature, the mixture was incubated at a range of temperatures at 60, 63, 65, 66, 67 and 68 °C for 50, 60, and 70 min. Subsequently, the mixture was incubated at 80 °C for 5 min to terminate of the reaction. For each reaction, a negative control (double distilled water) and positive control (*T. gondii* RH strain) were included.

The specificity of the *T. gondii* LAMP primers was examined using DNA derived from *Neospora caninum*, *Leishmania major* and *Trichomonas vaginalis*. A negative control without any template was included in each reaction. The detection limit of the LAMP and PCR was tested using serial tenfold dilutions of *T. gondii* DNA. The initial concentration of DNA for serial dilution was estimated using nanodrop. Furthermore, detection limit of LAMP and PCR were compared for detection of *T. gondii* DNA using samples of sparrows.

Analysis of the amplification products

The LAMP and PCR products were electrophoresed in a 2 % agarose gels stained with safe stain (CinaGen, Iran) and visualized under UV light. Visual inspection of the LAMP amplicons in the reaction tube was performed by adding 1 ml LAMP products to 10 µL of ethidium bromide stained solution (1 µg/ml) (Qiao et al. 2007).

Results

Detection of LAMP product

The LAMP successfully amplified *Toxoplasma* DNA under isothermal conditions. Optimum results were observed when the LAMP reaction was performed at 65 °C for 60 min. Agarose gel electrophoresis of the LAMP product showed ladder like patterns with multiple bands (Fig. 1). The direct visualization of LAMP amplicons using ethidium bromide stain was observed under UV transilluminator (302 nm) (Fig. 2a). A detectable yellow color pattern

Fig. 1 Agarose gel electrophoresis of LAMP (a) and PCR (b) products. Lane M, 100 bp DNA marker; PC positive control, PS positive sample. NC negative control

was observed in positive reaction and a red color pattern was shown in negative reaction (Fig. 2b).

The specificity of LAMP was tested using DNA of other parasites (*N. caninum*, *L. major* and *T. vaginalis*). As a result, the LAMP method was amplified only *T. gondii* DNA. Conventional PCR targeting the 529-bp repetitive element was also amplified only *T. gondii* DNA. The detection limit of LAMP was performed using serial dilutions of *T. gondii* DNA. The detection limits for the LAMP and PCR assays were 1 and 10 pg/μL respectively.

Among the 200 sparrow samples, 17 (8.5 %) and 15 (7.5 %) samples were positive by LAMP and conventional PCR, respectively (Figs. 1, 2). All of the PCR-positive samples were also positive by LAMP.

Discussion

In our study, the LAMP was successfully amplified *Toxoplasma* DNA at 65 °C for 60 min. We observed that the specificity of LAMP was similar to conventional PCR, while the LAMP and conventional PCR methods amplified only *T. gondii* DNA and no cross-reaction with non-targeted DNA from other parasites were found. The results showed that the detection limit of LAMP was higher than conventional PCR. While in a serial dilution of *T. gondii* DNA, the detection limits of the LAMP and PCR assays were 1 and 10 pg/μL respectively. In clinical samples, *T. gondii* was detected from 8.5 to 7.5 % of 200 sparrows by LAMP and conventional PCR, respectively. To our knowledge, this study is the first report of detection of *T. gondii* by LAMP method in bird hosts. Several reports were confirmed the higher sensitivity of LAMP for detection of *T. gondii* than conventional PCR targeting 529 bp fragment of *T. gondii* genome (Fallahi et al. 2014; Kong

Fig. 2 a Visual detection of LAMP under a UV transilluminator (302 nm) with black-with pattern (a), and color pattern (b). PC positive control, PS positive sample. NC negative control

et al. 2012; Lin et al. 2012; Zhang et al. 2009) or other specific targeting of *T. gondii* DNA regions (Fallahi et al. 2014; Hu et al. 2012; Kong et al. 2012; Krasteva et al. 2009; Lau et al. 2010; Lin et al. 2012; Sotiriadou and Karanis 2008; Zhang et al. 2009). Until now, LAMP method was used for detection of *T. gondii* in pig (Lili et al. 2014; Lin et al. 2012; Qu et al. 2013; Wang et al. 2013; Zhang et al. 2009), sheep (Lin et al. 2012), mice (Hu et al. 2012; Kong et al. 2012; Krasteva et al. 2009), and human (Fallahi et al. 2014; Lau et al. 2010). LAMP was also used for detection of *T. gondii* oocysts in water samples (Gallas-Lindemann et al. 2013; Koloren and Demirel 2013; Sotiriadou and Karanis 2008).

There are some reports on molecular frequency of *T. gondii* in bird hosts in Iran. In this regard, (Khademvatan et al. 2013) could detect *T. gondii* DNA from 15.5 % of 103 free-ranging chickens (*Gallus domesticus*), 6.9 % of 43 pigeons (*Columba livia*), 12.8 % of 39 starlings (*Sturnus vulgaris*), and 26.5 % of 64 sparrows (*Passer domesticus*) by PCR technique in Khuzestan province (southwest of Iran) (Khademvatan et al. 2013). Zia-Ali et al. (2005) detected *T. gondii* DNA from six out of 46 free-ranging chickens (13.4 %) and one out of 13 ducks (7.7 %) in Mazandaran province (northern Iran) (Zia-Ali et al. 2005). Asgari et al. (2009) detected anti-*Toxoplasma* antibodies in 58 out of 231 free-ranging chickens (25.1 %) in Fars province (Southern Iran). Then, 29 seropositive tissue samples were selected for molecular detection of *T. gondii* by PCR. Out of 29 seropositive tissue samples, *T. gondii* DNA was detected in 27 livers, 25 brains, and 16 heart samples (Asgari et al. 2009). Sarkari et al. (2014) detected anti-*Toxoplasma* antibodies in 89.8 % of 54 turkeys and *T. gondii* DNA in 61.6 % of seropositive turkeys in Fars

province (Sarkari et al. 2014). Recently, Tavalla et al. (2013) isolated *T. gondii* DNA in 8.6 % (13/150) of soil samples in Tehran (Tavalla et al. 2013). Interestingly, this result is very similar to our report with 8.5 % infection in sparrows, and confirms that birds are one of the best indicators for environmental contamination of *T. gondii* oocysts.

Anti-*Toxoplasma* antibodies were also detected a range between 2.7 and 86 % among stray cats in different parts of Iran (Derakhshan and Mousavi 2014; Hooshyar et al. 2007; Mosallanejad et al. 2011; Raeghi and Sedeghi 2011; Sharif et al. 2009; Tehrani-sharif et al. 2014). Meat born toxoplasmosis has been considered a major source of *T. gondii* infection (Kijlstra and Jongert 2009). People in some parts of Iran consume the sparrow meat traditionally. Hence, consumption of sparrow meat may be an important source of *T. gondii* infection in cats and human in Iran.

Molecular frequency of *T. gondii* in sparrows was also reported in Brazil (Gondim et al. 2010; Vilela et al. 2011) and China (Cong et al. 2013; Huang et al. 2012). In this regard, Gondim et al. (2010) detected anti-*Toxoplasma* antibodies in 1.02 % (3/293) and *T. gondii* DNA from 17.5 % (7/40) of sparrows in the northeast of Brazil (Gondim et al. 2010). Vilela et al. (2011) reported 60.3 % (90/51) *T. gondii* seropositivity among sparrows in region of Pernambuco, Brazil. They also detected *T. gondii* DNA from four sparrows by Nested-PCR (Vilela et al. 2011). Cong et al. (2013) detected anti-*Toxoplasma* antibodies among 12.46 % (39/313) of house sparrows in northwestern China. Moreover, *T. gondii* DNA was detected in 11 seropositive birds (Cong et al. 2013). In another study in China, *T. gondii* DNA was detected in four out of 178 wild birds (including 98 pheasants and 80 sparrows) (Huang et al. 2012). In a previous study, *T. gondii* antibodies were detected in 12.3 % of 227 house sparrows (*Passer domesticus*) and 4.9 % of 41 tree sparrows (*Passer montanus*) in Poland and the Czech Republic (Literák et al. 1997). *T. gondii* was also isolated in 0.5–40 % of sparrows from different regions of Europe [reviewed by (Dubey 2002)]. Experimental infection of house sparrows by oocysts of *T. gondii* showed a peak of antibody production at 7 weeks post-infection and reduction of the antibody titers at 12 weeks post-infection. These results demonstrated that sparrows do not produce a significant antibody in response to *T. gondii* (Literák et al. 1999).

Taken together with our results and other reports about high sensitivity and specificity of LAMP, this method can be used as an alternative assay for detection of toxoplasmosis. The prevalence of *T. gondii* infection in ground-foraging birds (like sparrows) has epidemiological significance, because indicating environmental contamination with *T. gondii* oocysts. Moreover, infected birds are potential reservoirs for *T. gondii* transmission to human and

cats. Therefore, the results of the present study provided an insight into epidemiological pattern of *T. gondii* infection in house sparrows in Iran.

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Conflict of interest The authors have no conflicts of interest.

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