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Genetic Characterization of *Toxoplasma gondii* Isolated from Chicken Meats in Tunisia

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Abstract

Background: Toxoplasmosis is one of the most important food-borne diseases distributed worldwide. In order to determinate the involvement of poultry meat in clinical toxoplasmosis, this study aimed to genetic characterization of *Toxoplasma gondii* in tissue samples of serologically positive chickens of Tunisia using Polymerase Chain Reaction (PCR).

Methods: Trail of isolation of local *T. gondii* chicken strains through bioassay of the suspected infected chicken tissues in mice was carried out and the isolated strains were typed using PCR-RFLP targeting the marker *AK69*. Data analysis was performed by SPSS 16.0.

Results: Out of 40 seropositive chickens, 15 were positive by *AK69* gene PCR. RFLP analysis showed high prevalence of type II *T. gondii* in infected poultry meats in Tunisia.

Conclusion: It seems that chicken meat of this region is not a risk factor of congenital toxoplasmosis. However, direct genotyping approach and deeper analysis should be investigated to have clearer idea about the epidemiology of the parasite and its transmission routs in Tunisia.

Introduction

Toxoplasma gondii, belonging to apicomplexa, infects humans and a wide range of mammals and birds (Dubey, 2010). Parasitic infection in human beings happen by consumption of undercooked meat harboring tissue cyst and/or by ingestion water or foods contaminated with oocysts. Naturally, most infected humans are asymptomatic and only a small percentage of infected humans show clinical signs of the disease. It seems that various strains involve in these different signs (Robert-Gangneux and Dardé, 2012).

The incidence rate of *T. gondii* varies in human among countries relating to contamination of the environment with oocysts expelled from cat, the definitive host (Dubey, 2010; Dubey et al., 1993). For detection of the various strains in environment, identification of *T. gondii* in chickens can be one of the best way because they are infected by feeding from soil polluted with oocysts (Ruiz and Frenkel, 1980). On the other hand, chickens are considered as source of this pathogen for human infection. Indeed, consumption of undercooked meat of free-range chickens might be an important source of in-

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fection for human beings (Boughattas and Bouratbine, 2014).

The most isolated *T. gondii* from human and animals are grouped into three clonal lineages by molecular techniques (Ajzenberg et al., 2002a; Ajzenberg et al., 2002b). The differences in sequencing among these three lineages are less than 1% but their virulence is very different. In contrast of type I, types II and III are less virulent (Sibley and Boothroyd, 1992). Finding the highest prevalent of these types in environment may help increasing our knowledge about the risk of human infection and related manifestations (Boothroyd and Grigg, 2002).

Our previous studies showed that there is a high prevalence of toxoplasmosis in commercial free-ranging chickens of Tunisia (Boughattas and Bouratbine, 2014) but we have no data about the types of this parasite (Boughattas et al., 2014). The aim of this study was to characterize *T. gondii* isolated from naturally infected free-range chickens slaughtered for human consumption in Tunisia.

Materials and methods

Bioassay

Chicken meats (forty) were processed as described elsewhere (Boughattas and Bouratbine, 2014) and each one was inoculated to mice in triplicate. Four weeks after inoculation, surviving mice were euthanized by cerebral dislocation according to ethical committee guideline in our university. If strains caused acute toxoplasmosis and followed by death of mouse within 10 days after inoculation, they were considered as virulent. If strains lead to asymptomatic chronic toxoplasmosis, they were grouped as avirulent. The strains resulted in sub-acute infection, were considered as intermediate virulence. Brains were collected and bloods were centrifuged to sample sera. Sera samples were examined for the presence of antibodies (IgG) against *T. gondii* by the modified agglutination test (MAT). Sera were assayed at a screening dilution of 1:20 and 1:200. A positive result at 1:20 or 1:200 or both was considered indicative of *T. gondii* exposure.

Brains of all mice were examined for tissue cysts as described by Dubey et al. (1993). The brains were removed, homogenized in 1 ml phosphate buffer saline (PBS, pH 7.2) by syringe as well as needle and then, examined for tissue cysts. For detecting the numbers of cysts in brain, each 20 µl of three aliquots were used for counting the cysts by microscope using 10X objective lens. After that, they summed and converted to a count per mouse brain. If it was observed at least one cyst in each inoculated mice or sera with MAT, the bioassay test was considered positive.

Molecular analysis

Specific primer pairs used in a PCR assay to amplify the polymorphic region of *AK69* gene include AK69Fex 5'-TTGAACATCTGGTGCGAGAC-3' and AK69Rex 5'-GTCTCCCAACCACCTCCATA-3' as external primers and AK69F 5'-ACGAGCAACCATATCTTACC-3' and AK69R 5'-CGAACGGACAACAAGCTA-3' as internal ones. The amplified reaction for the first round was performed by denaturation at 94 °C for 1 min, annealing at 55 °C for 50 s, and extension at 72 °C for 1 min. The second round was done the same as first round with different annealing temperature at 58 °C. As the template, a 1:100 dilution of PCR products from the first round was used for the second round (Boughattas et al., 2011). After checking of success amplification by agarose gel electrophoresis, RFLP analysis was done using digestion of *AK69* amplified products with *HinfI* as recommended protocol by the supplier (Invitrogen). The restriction fragments polymorphism was analyzed by 3% agarose gel electrophoresis alongside by DNA ladder.

Results

From the forty seropositive chickens in our previous work, we were able by the end of the mouse bioassay to detect *Toxoplasma* in 15 mice tissues (37.5%).

All the positive samples were also positive after *AK69* PCR amplification by the visualization of 490 bp fragment on agarose gel (Fig. 1). By the RFLP analysis used to genotype *T. gondii* strains, we did not observe any type I strain or mixed strain involving this allele. The comparison of chicken profiles obtained after enzymatic restriction to reference clonal strains (I, II and III), allowed to observe sample of genotype III, mixed genotype II/III in two cases, and the abundances of genotype II in the rest of samples (Fig. 2).

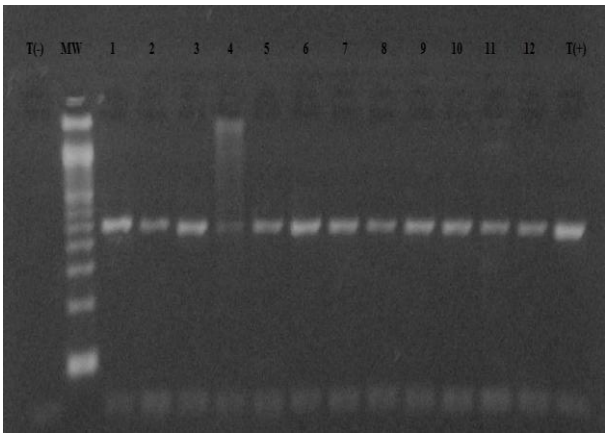

Fig. 1: Agarose gel electrophoresis after PCR targeting *AK69* marker. T (-): Negative test, MW: 100 bp DNA ladder, lanes 1-12: chicken samples and T (+): positive test

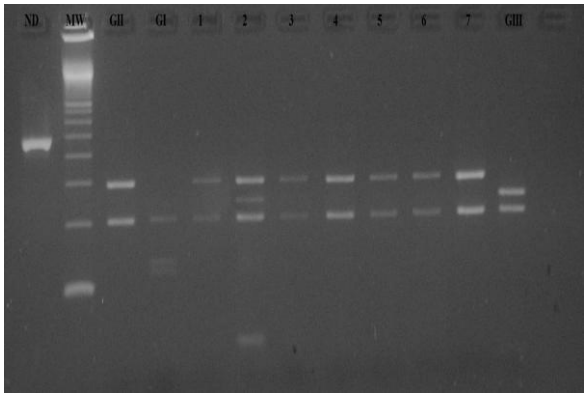

Fig. 2: Agarose gel electrophoresis after PCR-RFLP targeting the *AK69* marker. ND: non digested test, MW: 100 bp DNA ladder, GII: reference genotype II, GI: reference genotype I, lanes 1-7: restriction profiles of chicken samples (lane 2 showed complex profile of mixed genotype II/III), GIII: reference genotype III

Discussion

Earliest studies of molecular identification of *T. gondii* strains indicated that they have smaller genetic complexity than expected (Darde et al., 1992; Sibley and Boothroyd, 1992). Other teams showed that *T. gondii* strains could be categorized in three types I, II, and III (Howe and Sibley, 1995). Although these types have small differences in their sequences, their virulence is very different in hosts. Distribution of these types is related to region in which the host is reared. Studies on SAG2 or GRA6 showed that type II is prevalent in North America and Europe (Darde et al., 1992; Howe and Sibley, 1995; Howe et al., 1997), types I and III in Portugal and Spain (Fuentes et al., 2001; de Sousa et al., 2006), and type III in Crete and Cyprus (Messaritakis et al., 2008). In our previous study, we showed that seroprevalence of *T. gondii* was high in chicken samples (Boughattas et al., 2014). In our knowledge, there is no information about type dominant in chicken as foodborne and public importance (Barakat et al., 2012), and also for detection of dominant type in Tunisia.

Our previous analyses enable to state that the free-ranging chickens can be an important risk factor for pregnant women due to the high observed seroprevalence (Boughattas and Bouratbine, 2014). Recent study by Geuthner et al. (2014) detected that *T. gondii* may persist in poultry especially in Turkey. They showed that edible tissues may harbor the viable cysts for at least 16 weeks. This could increase potential risk for humans to be infected by consumption of undercooked avian meat. Therefore, it could be considered as a source of foodborne *T. gondii* for pregnant women. But, the types of *T. gondii* strains in chicken have not been identified in this region. To determine the types of toxoplasmosis cases in Tunisia for assessing the potential risk of the free-ranging chicken for pregnant women, molecular genotyping analysis were investigated. First, the mouse bioassay was performed for detecting the pathogenecity of *Toxoplasma* strains.

In the present study, we did not detect any genotype I strain or any mixed strain involving allele 1. The observed mixed genotype II/III was confirmed by multilocus microsatellites analysis (data not shown) as described elsewhere (Ajzenberg et al., 2005). The similar results were detected by Dubey et al. (2005). Dubey et al. (2003) also found the same results in Parana from Brazil. Recently, some studies showed that more types could find in *T. gondii* if more markers would be studied (Dubey et al., 2010; Verma et al., 2015). Surprisingly, genotype II was abundantly observed among chicken isolates. This genotype is frequently isolated in Europe. This finding does not match our previous observation in genotyping Tunisian isolates (Boughattas et al., 2014). It may be explained by the fact that previously, typing was investigated directly from samples without the bioassay approach. It is believed that mice may select some strains in favor of other to assure its survey (Villena et al., 2004). So, strain type II could be more frequently observed after mice inoculation as it seems to be the less pathogenic strain.

Conclusion

In conclusion, using the most sensitive PCR-RFLP marker, we were able to observe frequency of genotype II in free-ranging chicken in Tunisia. This could discard this type of meat of being risk factor of congenital toxoplasmosis. However, direct genotyping approach and deeper analysis should be investigated to have clearer idea about the epidemiology of the parasite and its transmission in Tunisia.

Conflicts of interest

The authors declare that there is no conflict of interest in this study.

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