Genetic and virulence characterization of *Toxoplasma gondii* strains isolated from pigeons in Lisbon region

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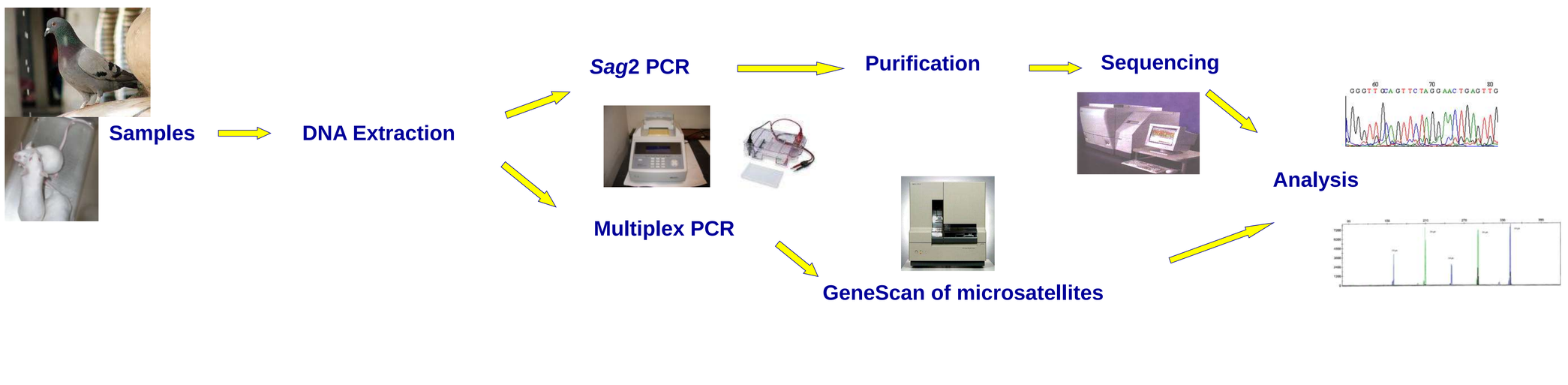
INTRODUCTION

Toxoplasma gondii is an apicomplexan obligate intracellular parasite and the most extensively studied of the tissue encysting coccidia. It has been estimated that one third of the world population has been infected **(Dubey 2008e, Su 2003, Tenter 2000)**. In most human adults it does not cause serious illness, however, blindness and mental retardation can be caused in congenitally infected children and severe diseases in those with compromised immunity **(Luft 1992, Montoya 2004)**. A recent study indicated that infection with *T. gondii* is also associated with abdominal hernia **(Alvarado 2011)** and could increase the risk of brain cancer because it is a long-lived parasite that encysts in the brain, where it provokes inflammation and inhibits apoptosis **(Thomas 2011)**. *T. gondii* has been differentiated in three major groups, type I, II and III **(Dardé 2003, Howe 1995, 1997, Sibley 1992)**. In pigeons, different prevalences have been described, ranging from 4,6% to 100% **(Cotteleer 1978, Kirkpatrick 1990, Mushi 2000, Tsai 2006, Waap 2008, Salant 2009, Yan 2011)**. In Portugal there is only a preliminary study in pigeons typing developed by our work group **(Waap 2008)**. The microsatellite typing revealed that 75% of strains belonged to type II, 16,7% were type III and 8,3% was type I **(Waap 2008)**. This work is part of a global study which aim is to help the understanding of the portuguese reality that concerns *Toxoplasma gondii* typing and virulence strains.

METHODS

Sample – 41 brains of pigeons which showed positive serology for *T. gondii*.
All brains were inoculated in mice and maintained for 3 months. Serology was performed in mice at 15 days, 1 month, 2 months and 3 months after inoculation. *T. gondii* DNA was extracted by columns with proteinase K from pigeons and inoculated mice brains. The two ends of *Sag*2 gene were amplified by PCR and held sequencing **(Howe 1997)** in both type of samples. The sequencing of *Sag*2 can't identify recombinant or atypical strains, therefore we also amplified 5 microsatellites regions (TUB2, W35, TgM-A, B18 and B17) by multiplexPCR and detected the length polymorphisms of microsatellites by GeneScan **(Fig. I)**. Microsatellites are more discriminative and allows the identification of these recombinant or atypical strains **(Ajzenberg 2002)**.

Fig. I – Works diagram



RESULTS

The isolation rate in mice of the 41 brain tissue was 58,5% (n=24). The 24 mice inoculated had antibodies (ab) Anti-*T. gondii* at 15th day after inoculation with one exception, which showed positive serology within 30 days after inoculation. None of the isolates were virulent to the mouse. In all mice with positive serology for *T. gondii* we performed a "post-mortem" macroscopic analysis of the organs (brain, liver, lungs, heart and spleen), in which all had a normal conformation. We also performed a microscopic analysis where we observed brain cysts. *T. gondii* DNAs were extracted (when present) from brain homogenates of the 41 pigeon and held the B1 gene PCR for the parasite identification. We obtain amplification in 29 of the 41 pigeons brains, which showed a *T. gondii* identifying rate of 71%. The remaining 12 samples were negative and was not observed any inhibition. Of the 29 positive samples to the B1 sequence gene, the genotyping by *Sag*2 gene was achieved in 22 samples, for one of the three genotypes. 19 samples belong to type II, 2 to type III and 1 to type I. In other 5 samples we can't amplify one of the two ends of *Sag*2 gene revealing the other end of *Sag*2 gene that strains belonging to type I or to type II. The 2 remaining samples were not possible to genotype, possibly due to low concentrations of DNA. Unlike the amplified B1 gene sequence, the sequences of *Sag*2 gene are single copy gene and the 3' fragment is more difficult to amplify possible due to its nucleotide constitution. Microsatellites proved quite difficult to amplify, possible due to the low DNA concentration or from the organic matrix of the initial product. However, it was possible genotype 22 samples, by these technique. They were not amplified as a 5 multiplex PCR, but 3 multiplex PCR and the other 2 microsatellites were amplified separately. Genotyping by microsatellites showed concordance with previous results of the genotyping of the two ends of *Sag*2 gene. The differentiation of strains was also performed in the brains of positive inoculated mice, being consistent with differentiation performed directly from the biological product (brain pigeon). Nevertheless, the differentiation by *Sag*2 gene of these products revealed easier to perform than using the primary organic pigeons products, probably because the inoculation in mice enhances the strains concentration.

DISCUSSION AND CONCLUSIONS

The pigeons shares the same habitat with cats and humans, bands are observed in recreational areas such as urban parks, playgrounds and parks. The interaction between cats, birds and human population is quite evident favoring the fecal-oral transmission of *T. gondii* between the definitive host and intermediate hosts, in the urban cycle of the parasite. The results of the inoculation in vivo of the brain homogenates showed pigeon isolation rates (58.5%) significantly higher when compared with previous studies, including the preliminary study in 2006 that the isolation rate in mice was 39,1% (9/23) **(Waap 2008)** and another that was not achieved any isolation in mouse **(Godoi 2010)**. The genotypic analysis revealed a majority of strains of type II, which is consistent with what has been described in Portugal, the rest of Europe and the USA **(Ajzenberg 2005, Fazaeli 2000, Honoré 2000, Howe 1997, Waap 2008)** . We also isolated strains of type III and type I. The identification of type III strains in animals have been reported by other authors, but the type I have been rarely found in animals has not been previously described in Portugal except in a preliminary study of our team at the 2008 **(Waap 2008)**. The type I strains are usually associated with high virulence in laboratory mice, leading to death within days. This strain was identified by molecular biology and has not been isolated in vivo. The difficulty in isolation of strain may be related to the small number of cysts of the type I strains can develop, these type strains are considered low cystogenic. Genetic characterization of strains of *T. gondii* is far from its terminus, more sequences of different genes should be studied to help the understanding of the molecular epidemiology and genetic characterization of *T. gondii*, a relevant parasite for which these data are lacking. The combination of data from humans and animals, through the use of high resolution genetic characterization should improve our perceptive of *T. gondii*, which will be ultimately beneficial for the control of *T. gondii* transmission.

REFERENCES

Ajzenberg D, Banuls A, Tibayrenc N and Dardé ML. 2002. Microssatellite analysis of *Toxoplasma gondii* of population shows a high polymorphism structured into two main clonal groups. Int J Parasitol. 32:7-38.
Ajzenberg D, Dumètre A and Dardé ML. 2005. Multiplex PCR for typing strains of *Toxoplasma gondii*. J Clin Microbiol. 43:1940-3.
Alvarado-Esquivel C and Estrada-Martínez S. 2011. *Toxoplasma gondii* infection and abdominal hernia: evidence of a new association. *Parasit Vectors.* 4:112.
Cotteleer C and Famerée L. 1978. Intestinal parasites and anti toxoplasma antibodies in the pigeons of Belgium. Schweiz Arch Tierheilkd. 120:181–7.
Dardé ML. and. Ajzenberg D. 2003. Toxoplasmosis polymorphism and its clinical consequences. Archives de pédiatrie. 10:45-6.
Dubey JP. and Jones JL. 2008. *Toxoplasma gondii* infection in humans and animals in the United States. Int J Parasitol. 38:1257–78.
Fazaeli A, Carter P, Dardé ML and Pennington T. 2000. Molecular typing of *Toxoplasma gondii* strains by GRA6 gene sequence analysis. Int J Parasitol. **30:**637-642.
Godoi F, Nishi S, Pena H and S. Gennari. 2010. *Toxoplasma gondii*: diagnosis of experimental and natural infection in pigeons (*Columba livia*) by serological, biological and molecular techniques. Rev Bras Parasitol Vet. 19(4):238-43.
Honoré S., A. Couvelard, Y. Garin *et al.* 2000. Génotypage des souches de *Toxoplasma gondii* chez des patients immunodéprimés. Pathologie Biologie. **48:**541-7.
Howe DK. and. Sibley LD. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis. 172:1561–6.
35:1411–4.
Howe D, Honoré S, Derouin F and Sibley L. 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J Clinic Microbiol. 35:1411-4.
Kirkpatrick C, Colvin B and Dubey JP. 1990. *Toxoplasma gondii* antibodies in common barn-owls (Tyto alba) and pigeons (Columba livia) in New Jersey. Vet Parasitol. 36:177–180.
Luft B and Remington J. 1992. Toxoplasmic encephalitis in AIDS. Clin Infect Dis. 15:211-22.
Montoya J and Remington J. 1996. Toxoplasmic chorioretinitis in the setting of acute acquired toxoplasmosis. Clin Infect Dis. 23:277-82.
Mushi E, Binta M, Chabo R, Ndebele R and Panzirah R. 2000. Parasites of domestic pigeons (Columba livia domestica) in Sebele, Gaborone, Botswana. J S Afr Vet Assoc. 71:249–50.
Salant H, Landau D. and Baneth G. 2009. A cross-sectional survey of *Toxoplasma gondii* antibodies in Israeli pigeons. Vet Parasitol. 165:145–9.
Sibley LD. and Boothroyd JC. 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature. 359:82–5.
SU C, Evans D, Cole RH, Kissinger JC, Ajioka JW and Sibley LD. 2003. Recent expansion of *Toxoplasma* through enhanced oral transmission. Science. 299:414–6.
Tenter A, Heckeroth A and Weiss L. 2000. *Toxoplasma gondii*: from animals to humans. Int J Parasitol. 30:1217-58.
Thomas F, Lafferty K, Brodeur J, Elguero E, Gauthier-Clerc M and Missé D. 2011. Incidence of adult brain cancers is higher in countries where the protozoan parasite *Toxoplasma gondii* is common. Biol. Lett. 8:101-3.
Tsai Y, Chung W, Lei H and Wu Y. 2006. Prevalence of antibodies to *Toxoplasma gondii* in pigeons (Columba livia) in Taiwan. J Parasitol. 92:871.
Waap H, Vilares A, Rebelo E, Gomes S and Ângelo H. 2008. Epidemiological and genetic characterization of *Toxoplasma gondii* in urban pigeons from the area of Lisbon (Portugal). Vet Parasitol. 157:306-9.
Yan C, Yue C, Qiu S. *et al.* 2011. Seroprevalence of *Toxoplasma gondii* infection in domestic pigeons (Columba livia) in Guangdong Province of southern China. Vet Parasitol. 177:371-3.

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