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Prevalence and genotyping of *Toxoplasma gondii* in pregnant women attending the Aristide Le Dantec University Hospital in Dakar, Senegal

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Abstract
Toxoplasma gondii is one of the most widespread parasites in the world that infects humans and other warm-blooded animals. It causes asymptomatic toxoplasmosis in immunocompetent adults while in pregnant women, primary infection can lead to congenital toxoplasmosis in foetuses and newborns. Therefore, in pregnant women, early and accurate diagnosis of toxoplasmosis can be crucial for the prevention and control of the disease. Routinely, immune status against *T. gondii* is achieved by identifying the parasite-specific antibodies in the serum with serological techniques such as Enzyme-linked Immunosorbent Assay. Another factor of the *T. gondii* infection severity in humans is the strain virulence of the parasite. Three genetically different types (strain) are described (type I, type II and type III) basing on the genetic analysis of the polymorphic surface antigen 2 locus (SAG2) by PCR-RFLP. Identification of the genetic type helps to better understand the disease and the possibly to find the appropriate treatment. Hence the aim of this study was to determine the lineage types of *T. gondii* in pregnant women diagnosed with positive serology against *T. gondii* in Senegal. From January to December 2016, 104 pregnant women attending the Parasitology-Mycology laboratory of Le Dantec University Hospital, Dakar were enrolled. Among them, 48 (46.2%) were found with IgG antibodies. B1 gene-PCR realized on these latter revealed 20 positive cases (41.7%), confirmed by 5′-3′ SAG2 gene-PCR. Enzymatic digestion of positive samples successively with the HhaI and the Sau3AI enzymes revealed 85% of type III and 15% of type I. In definitive, this study, first among pregnant women in Senegal showed that type III of *T. gondii* was predominant and none of the samples was type II.

Keywords: *Toxoplasma gondi*,; serology, genotyping, pregnant women, Senegal

1. Introduction
Toxoplasma gondii is an intracellular protozoan, considered as one of the most widespread parasites in the world that infects humans and other warm-blooded animals [1]. In immunocompetent adults, toxoplasmosis is mainly asymptomatic while in pregnant women, primary infection of *T. gondii* can lead to congenital toxoplasmosis in foetuses and newborns, including a wide range of manifestations (mild chorioretinitis, miscarriage, mental retardation, microcephaly, hydrocephalus and seizures) [2, 3]. Therefore, in risk patients, especially in pregnant women, early and accurate diagnosis of toxoplasmosis can be crucial for the prevention and control of the disease [2].
Routinely, immune status against *T. gondii* is achieved by identifying the parasite-specific antibodies in the serum with serological techniques such as Enzyme-linked Immunosorbent Assay (ELISA) and Immuno-fluorescence Antibody Assay (IFA) [2].
Another factor of the *T. gondii* infection severity in humans is the strain virulence of the parasite [1]. Three genetically different types (strain) of *T. gondii* are described (type I, type II and type III) basing on the genetic analysis of the polymorphic surface antigen 2 locus (SAG2) by PCR-RFLP. Because of their genetic differences, the three types also differ in the mode of infection and the severity of the symptoms. Therefore, identification of the genetic type helps to better understand the disease and the possibly to find the appropriate treatment [4].
The aim of this study was to determine the lineage types of *T. gondii* in pregnant women diagnosed with positive serology against *T. gondii* in Senegal.

2. Patients and Methods
2.1. Study site and sampling
From January to December 2016, as previously described [2] a cross-sectional study was conducted in the Parasitology-Mycology laboratory in Le Dantec University Hospital, Dakar region, Senegal using multi-stage methods. Initial stage involved enrolment of all pregnant women received at the laboratory for determination of immune status against *T. gondii*. Women were from different healthcare structures of Dakar, the capital.
The second stage was embracement of the laboratory investigation that included Enzyme-linked Immunosorbent Assay (ELISA), DNA extraction, PCR, nested-PCR assay, and genotyping of the seropositive cases.
From each consented participant, 5 ml of blood samples were collected. Each sample was divided into two parts of, 2ml in an EDTA tube for extracting the DNA and 3 ml in a plane tube kept to extract serum. The later was immediately

centrifuged for 5 minutes at 3000 rpm. Thereafter, the resulted serum was transferred then into a clean dry Eppendorf tube and kept in under -20 °C for further analysis.

2.2. Measurement of the specific antibodies against Toxoplasma gondii

All serums samples were tested using ELISA by Immuno-comb Toxo-IgG kit manufactured by Orgenics Ltd, Yavne, Israel, with a sensitivity of 97.2% and specificity of 93.75%. For interpretation, of positive control must produce 2 spots on the card tooth to be valid; negative control must produce only an upper control spot to be valid. A lower spot with an intensity higher than or equal to that of positive indicates the presence of IgG antibody to *Toxoplasma gondii*.

2.3. DNA Extraction

DNA was extracted from the venous whole blood using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, and USA) according to the manufacturer's instructions.

2.4. Detection of Toxoplasma gondii by nested-PCR

Presence of *T. gondii* DNA were confirmed by nested PCR amplification of the repetitive and conserved gene B1 [2] using B1 primers (Table 1). Briefly, it begun by 15 min of enzymes activation at 95 °C. Then, for the both amplifications, it involved 39 repeated cycles of: denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C for the first amplification and 60 °C for the second, and extension for 30 s at 72 °C. At the end of these cycles, a final extension was done for 10 min at 72 °C.

The second stage of amplification entailed a nested-PCR, which was performed to amplify the products of the first PCR cycle to obtain clear DNA. This was done by using 1 µL of the PCR products of the first cycle with the rest of PCR Master Mix contents plus the primers B1R2 and B1F2 at 60 °C for 30 s (Table 1).

Table 1: Primers used for amplification of the B1 gene

B1F1	**GGAACTGCATCCGTTCATGAG 5'-3'**
B1R1	TCTTTAAAGCGTTCGTGGTC 5'-3'
B1F2	TGCATAGGTTGCAGTCACTG 5'-3'
B1R2	GGCGACCAATCTGCGAATACACC 5'-3'

An 8 µl of each PCR product was subjected to gel electrophoresis in 3% agarose gel electrophoresis. The reaction is expected to yield a product of 97-100 bp. RH, Beverly and ME49 strains of *T. gondii* were used as positive controls, which was obtained from the parasitology and Mycology laboratory of Limoges University hospital, France.

2.5. Genotype analysis by PCR-RFLP

The positive cases for B1 gene samples were subjected to strain typing by nested-PCR-RFLP of the amplified SAG2 gene of *T. gondii*. The nested PCRs separately amplified 5′ and 3′ ends of the gene, followed by restriction enzyme application as previously described by Fuentes *et al*, [4].

The 5′ and 3′ ends of the gene were amplified using respectively 5' SAG2 1F (5'-GCT ACC TCG AAC AGG AAC AC-3') and 5' SAG2 1R (5'-GCA TCA ACA GTC TTC GTT GC 3') and 3' SAG2 1F (5'-TCT GTT CTC CGA AGT GAC TCC-3') and 3' SAG2 1R (5'-TCA AAG CGT GCA TTA TCG C-3') during the first amplification. In the second amplification, 5' SAG2 2F (5'-GAA ATG TTT CAG GTT GCT GC-3') and 5' SAG2 2R (5'-GCA AGA GCG AAC TTG AAC TT-3') and 3' SAG2 2F (5-ATT CTC ATG CCT CCG CTT C-3') and 3'SAG2 2R (5'-AAC GTT TCA CGA AGG CAC AC-3') were respectively used.

The cycling conditions were denaturation at 94°C for 5 min, followed by 40 cycles at 94 °C for 45 s, at 60°C for 1 min, and at 72°C for 2 min for each cycle. The final cycle was followed by an extension step at 72°C for 10 min.

Annealing temperature was varying according to used primers with 58 °C for the second amplification of the 5' SAG2 and the first amplification of the 3' SAG2 and then 55 °C for the second amplification of the 3' SAG2.

Yielded product for the 5′ SAG2 end amplification was at 241 bp, while for the 3′ SAG2
end amplification it was at 221 bp.

The amplified products were digested with Sau3AI enzyme (Sau3AI, catalog no. RO169S; New England BioLabs) and HhaI enzyme (HhaI, catalog no. R0139S; New England BioLabs), respectively for of the 5′ end and of the 3′ end, in order to determine the SAG2 type.

For interpretation, enzymatic digestion of the 5' end distinguishes type III from type I and II strains, whereas enzymatic digestion of the 3' end differentiates type II from type I and III.

RH, Beverly and ME49 strains of *T. gondii* were used as positive controls (obtained from the parasitology and Mycology laboratory of Limoges University hospital, France).

The digestion was conducted at a temperature of 37 °C overnight. The resulted digestives were run on 3% agarose gel.

2.6. Data description

Data were coded and entered into Microsoft Excel software (version 2016). They were described using frequencies and percentage.

2.7. Ethical consideration

The study was approved by the Ethics Committee of the Ministry of Health of Senegal. Participants were informed of the purpose and procedures of the study. Consent was obtained before taking the sample.

3. Results

3.1. Characteristics of the study population

In total, 104 pregnant women consented to participate in our study from the 141 received patients in the laboratory during the study period. It corresponds to a proportion of 73,8% of acceptance. They came from 15 localities of the capital (Dakar department) and its suburban area. Patients age ranged between 19 and 41 years.

3.2. Seroprevalence and genotyping

From the 104 pregnant women enrolled, 48 (46.2%) were found with antibodies against *T. gondii*. Among them, 40 were positive for IgG only (38.4%) and 8 for both IgM and IgG (7.69%). The mean age of these patients was 27 years. Among them, 34.9% were in their first trimester of pregnancy, 32.1% in their second trimester, and 33% in their last trimester.

B1-PCR realized on the 48 IgG positive samples revealed 20 positive cases (41.7%) and 4 equivocal cases. All positive samples showed an amplified product of 100 bp as yielded for the *T. gondii* B1 gene (Figure 1).

Fig 1: Nested PCR on B1 gene

PM: molecular weight markers 100 bp.
Wells: 1, 2, 3, 4, 7, 8, 9, 12, 14, 15, 16, 17, 18, 22, 29, 46, 49, 72, 74: positive samples.
Wells: 10,19,52,67: equivocal samples (in red).
Wells: CT1, CT2, CT3 : positive controls.
Wells: CN: Negative control.
The amplification of the SAG2 gene on all the 24 samples (positive and equivocal) confirmed that only the 20 previously positive for B1 were really positive (83%) whereas the 4 equivocal cases were revealed finally negative. Amplified products were 241 bp and 221 bp weighted respectively for the 5' and 3' end (Figure 2).

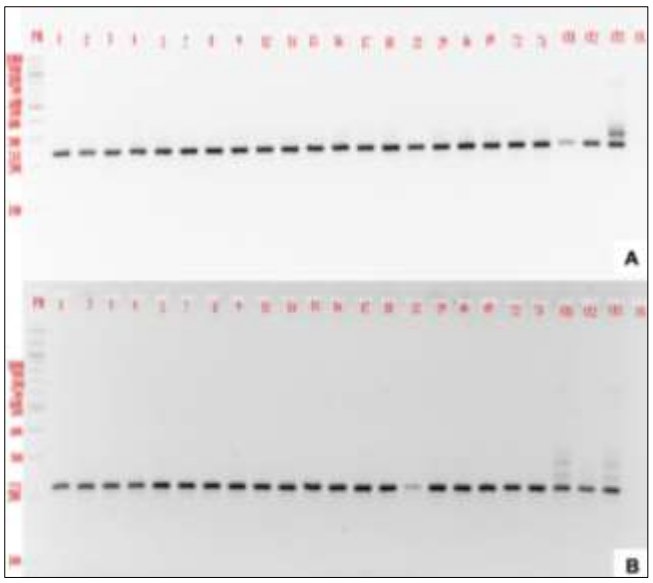

Fig 2: Nested PCR of the SAG 2 gene showing a 241 bp product at the 5' end (A) and a 221 bp product at the 3' end (B).

PM: molecular weight markers 100 bp
Wells: 1, 2, 3, 4, 5, 7, 8, 9, 12, 14, 15, 16, 17, 18, 22, 29, 46, 72 and 74: positive samples Wells: CT1, CT2 and CT3: positive controls.
Wells: CN: negative control
Thus, enzymatic digestion was realized on 20 samples (SAG2 gene positive). After a first incubation of the HhaI enzyme for the 3′ end, we did not find any type II positive sample (Figure 3).

Fig 3: Enzymatic HhaI restriction digestion of amplification products of the 3 'end

PM: molecular weight markers 100 bp
Wells: 1, 2, 3, 4, 5, 7, 8, 9, 12, 14, 15, 16, 17, 18, 22, 29, 46, 72 and 74: positive samples.
Wells: CT1, CT2 and CT3: positive controls
For distinguishing type I to type III, we incubated secondly the samples with the Sau3AI enzyme specific to the 5′ end and 17 samples (85%) were revealed to belong to the type III while 3 (15%) were type I (Figure 4).

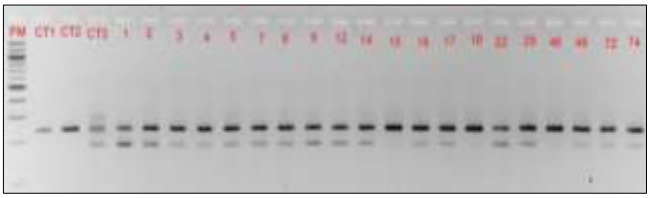

Fig 4: Sau3AI Enzymatic restriction digestion of 5' end amplification products

PM: molecular weight markers 100 bp
Wells 1, 2, 3, 4, 5, 7, 8, 9, 12, 14, 16, 17, 22, 29, 72 and 74: positive samples for type III; 15, 18 and 46, positive samples for type I.
Wells: CT1, CT2 and CT3: positive controls

4. Discussion

Studies about seroprevalence of toxoplasmosis are frequently reported in Africa and also in Senegal, especially among pregnant women. However, limited studies have been conducted to type the *T. gondii* circulating stains. Thus, the objective of this present study was to characterize genetically circulating stains of *T. gondii* from blood samples of pregnant women who present antibodies against this parasite.
Our serological findings showed a prevalence among pregnant women of 46.2%.
This result is not very far from that we found in our previous study conducted in the same laboratory during the period covering 2011 to 2014 with a prevalence of 40.03% [5]. Similar prevalence's were also found in Dakar by other authors with 43.8% by Ndour in 2012 [6] and 44.4% by Ndiaye in a study conducted between January 2010 and October 2012 [7]. In contrary, it was higher than prevalence found among pregnant women attending the military hospital of Ouakam, Dakar where Seck *et al*, [8] reported a prevalence of 32.70% in 2015.
The difference with this last resulted can be due to the difference according to the used techniques because Seck *et al*, had used a technique based on chemiluminescence.
Toxoplasma gondii is an interesting microorganism which led to a global effort to study its genetic diversity. It has been described as a parasite with low genetic diversity and a clonal population structure [9]. The first evidence for the existence of different strains of *T. gondii* came from isoenzyme analysis of isolates mainly of French origin. Parasite strains can be divided into three clonal lineages: types I, II, and III, according to their pathogenicity in mice [1]. Indeed, it is well known that the virulence of *T. gondii* differs in animals, depending on the *T. gondii strain* [4] and that congenital toxoplasmosis is associated mainly with type I and II strains. Most strains from patients with acquired immunodeficiency syndrome are type II, and animal strains are predominantly type III. It exists different methods of characterization, from isoenzyme electrophoresis to molecular methods such as restriction fragment length polymorphism (RFLP), PCR, or random amplified polymorphism DNA [4].
After use of B1-PCR, half of the samples were found

negative.

In Saudi Arabia, lesser proportion of B1-PCR positive samples was recorded with only 22.2% of positive samples [2].

This could be due to the fact that *T. gondii* is a tissue parasite with a very brief blood phase [10]. This is the reason why several other samples such as CSF, amniotic fluids, aqueous humor, and lung and brain biopsy have been used in some studies [4, 11].

Among these B1 gene positive samples, 4 were equivocal. However, using SAG2 gene, they were confirmed negative.

By using PCR-RFLP analysis, we found that 85% of our strains belong to the type III of *T. gondii*.

Similar results were obtained in Crete and Cyprus with 80% of type III [1]. Therefore, we noted a difference with our remaining results; 15% of our findings belonged to the type I whereas none of the samples in this study in Crete and Cyprus was type I.

In Saudi Arabia too, only two types were observed with 80.6% of type II and 19.4% of type III [2]. In contrary, in Spain, Fuentes *et al*, found the all three types with 40% of type I, 40% of type II and 20% of type III [4] whereas in Menoufia governorate, Cairo, Egypt, the determined genotype of *Toxoplasma gondii* isolates was type I [12].

These distributions seem logical since the type III found in all these studies is considered cosmopolitan while the type II absent from our study is mostly distributed in South America [13] even if it was found predominant in Saudi Arabia and in Iran [2, 14].

5. Conclusion

In definitive, our study, which is, to our knowledge, the first to characterize *T. gondii* strains genetically in pregnant women in Senegal, showed that type III was predominant and that none of our strains is of type II.

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