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Genotyping of *Toxoplasma gondii* samples from Dakar

Amadou Ndiaye[1], Pape Mbacké Sembene[3], Ngor Faye[3], Daouda Ndiaye[4], Daniel Ajzenberg[5], Niama Diop Sall[1,2] and Ben Sikina Toguebaye[3]

[1]Department of Laboratories Abass NDAO Hospital, Dakar, Senegal.
[2]Faculty of Medicine, Pharmacy and Dentistry (FMPOS), Dakar, Laboratory of Medical Biochemistry., Dakar, Senegal
[3]Department of Animal Biology, Faculty of Science and Technology, Dakar – Senegal
[4] Department of Parasitology and Mycology Pharmacy and Dentistry (FMPOS), Faculty of Medicine, Dakar, Senegal.
[5]National Reference Center for Toxoplasmosis, Laboratory of Parasitology and Mycology, Centre Hospitalier - Universitaire Dupuytren, 2 avenue Martin Luther King, 87042 Limoges, France.

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Toxoplasmosis is an anthropozoonosis of medical and veterinary importance, caused by the protozoan *Toxoplasma gondii*. Oocysts shed by felids play a key role in parasite transmission as they contaminate meat-producing animals, vegetables and water consumed later by humans. In this work, we aimed to identify *T. gondii* isolated in Dakar (Senegal). The modified multiplex PCR technique based on length polymorphism of 15 microsatellite markers was used on a total of 10 isolates. The study shows that stem called Africa 1 or Type I / III (90%) and Africa 2 or Type I / II / III (10%) circulate in Dakar. Toxoplasma isolates and DNA extraction for genotyping analysis was performed directly on clinical samples for 56 patients and indirectly on infected mouse tissue (brain or ascitic fluid) or infected cell cultures after inoculation of clinical samples for 32 patients. These first results need to be followed by more extensive investigations.

Key words: Genotype, toxoplasmosis, *Toxoplasma gondii*.

INTRODUCTION

Toxoplasma gondii (T*. gondii*) is the agent of a cosmopolitan anthropozoonosis: toxoplasmosis. This intracellular parasite maintains an optional heteroxenous cycle between cats (definitive hosts) and other warm-blooded animals (intermediate hosts). Toxoplasmosis is almost always asymptomatic but can be severe in immunocompromised individual or after congenital transmission. The medical and veterinary importance of toxoplasmosis drives for 50 years numerous epidemiological studies to identify the reservoirs and modes of transmission of the parasite (Try et al., 2000). The consumption of raw or undercooked meat containing cysts of the parasite and the ingestion of oocysts with fruits and contaminated with faeces of cats vegetables are the two main modes of contamination. More recently, the consumption of water contaminated with oocysts was identified as a risk factor for toxoplasmosis in Brazil (Bahia - Oliveira et al., 2003). Waterborne outbreaks have been causing symptomatic toxoplasmosis sometimes fatal Panama (Benenson and et al., 1982), in Canada (Bowie et al., 1997) and Brazil (Tavern, 2002). The seroprevalence of human toxoplasmosis varies according to geographical areas. In Europe, it is 30 to 50% in the majority of countries in central and west and becomes less than 30% in the north. Low

*Corresponding author. Email: ndiaydoc@live.fr, drndiayeamadou1@yahoo.fr. Tel: +221 33 849 78 41/221 33 849 78 39. Fax: + 221 33 842 00 23.

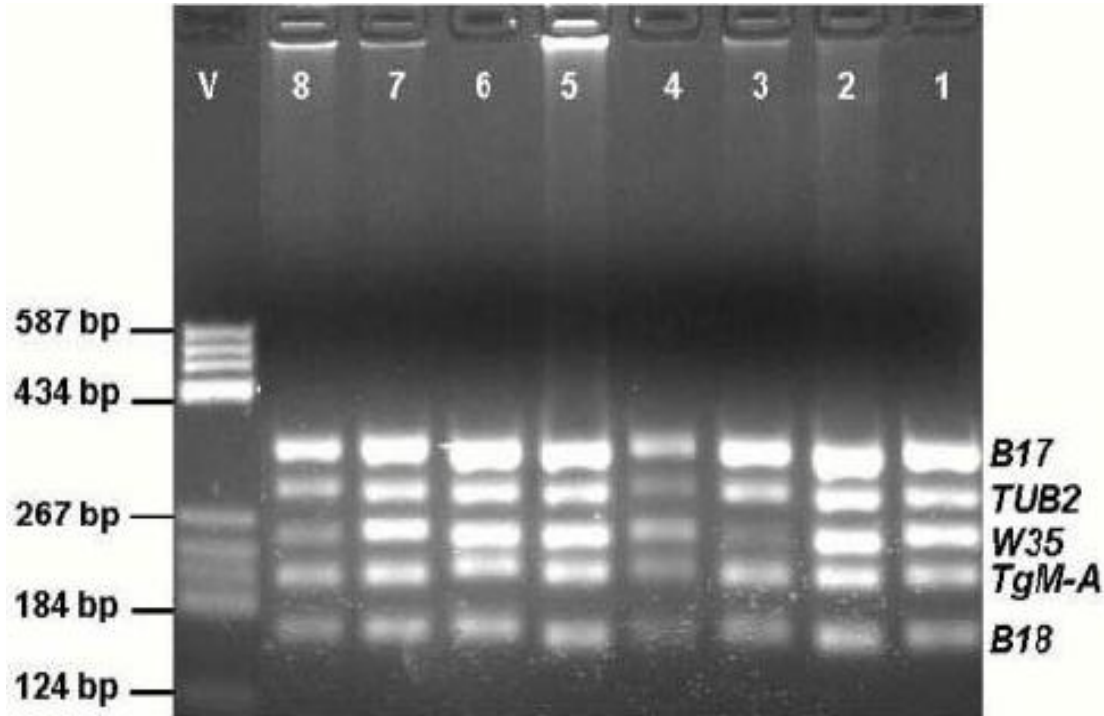

Figure 1. Example of agarose gel electrophoresis after amplification of five microsatellite markers by multiplex PCR (Ajzenberg et al., 2005) . Tracks, isolates: 1, BK; 2, ME49; 3, NED; 4, BOU; 5, DEG; 6, RMS-2001-MAU; 7, PSP-2003-KOM; 8, GUY-2002-MAT; V, molecular weight marker.

prevalences are recorded in North America, Southeast Asia and some African countries (Niger, South Africa) (Try et al., 2000). The highest prevalence (> 60 %) occurs primarily among the countries bordering the Gulf of Guinea and Latin America. These differences are mainly due to the larger survival of oocysts in humid climates. There are few infections in areas where cats are absent (Dubey, et al., 1997).

Oocysts have a central role in transmission of the parasite because they infect humans directly or indirectly through animals for slaughter. From the perspective of assessing the risk of toxoplasmosis associated with oocysts, it is necessary to determine the prevalence of oocysts in the environment. This is only possible with methods specific and sensitive to detection because the probability of isolating oocysts in naturally contaminated random sample is very low. The study of prevalence of toxoplasmosis in herbivores is also an interesting way to indirectly assess the prevalence of oocysts in the environment.

The work presented here is part of our research for our doctoral thesis and is in quite recent concern to identify strains of *T. gondii* circulating in Dakar, Senegal among others. It describes the nature of strains isolated at Dakar compared to the reference strains using a technique borrowed from Dr. Daniel Ajenberg namely multiplex PCR strains of Toxoplasma. The objective was to identify *T. gondii* samples isolated in Dakar (Senegal).

MATERIALS AND METHODS

All animals were tested in advance by ADHS. The blood was collected in a dry tube: The jugular vein in sheep, cattle and chickens (after decapitation); the heart chamber for wild animals killed or euthanized (if serology could be made from cardio -thoracic fluid).

The parasite was then investigated in animals infected by pepsin digestion of the brain and/or heart according to the method of Dubey (1998c).

It consists of the extraction of DNA after experimental infection of strains from human toxoplasmosis by pepsin digestion of the brain and/or heart according to the method of Dubey (1998c). Finally, a multilocus typing was done after amplification of sequences by multiplex PCR (Ajzenberg et al., 2005) (Figures 1 and 2)

Genetic typing of isolates of toxoplasma was based on the analysis of allelic polymorphism of five microsatellite markers: Tub2, W35, TgM -A, B18 and B17 (Ajzenberg et al., 2002a, 2004, 2005). Their combination allows a very resolutive typing; can highlight allelic recombination, not detected by the conventional method of typing SAG2 single locus by PCR -RFLP. Typing is performed after amplification of sequences by multiplex PCR (Ajzenberg et al., 2005). DNA was extracted from the brain or ascites fluid of mice infected with the animal isolates with the kit QIAamp ® DNA Mini Kit (Qiagen, Courtaboeuf,France).

The primers were synthesized by Applied Biosystems (Courtaboeuf, France). For each primer pair, one primer was coupled to the 6 - carboxyfluorescein (6FAM) or hexachloro -6-carboxyfluorescein (HEX) to the 5 'end to determine the length of the PCR products of automated sequencer. PCR was performed

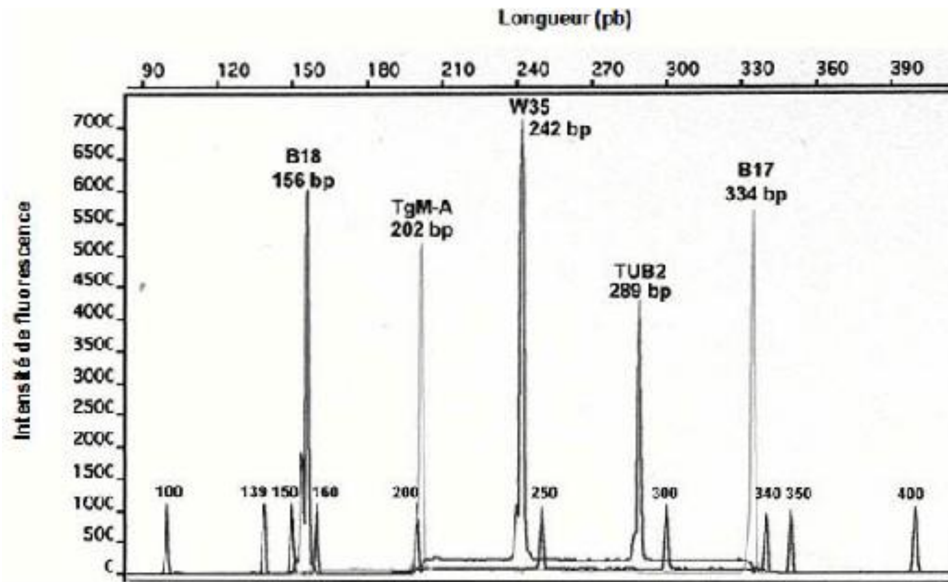

Figure 2. Example of electrophoresis of multiplex PCR products of genotype II (ME49) on automatic sequencer (Ajzenberg et al., 2005).

Table 1. Result by genotype found.

Genotype	Number	
	Absolute value	Relative value (%)
Type I	0	0
Type II	0	0
Type III	0	0
Type I/II	0	0
Type I/III (Africa1)	9	90
Type I/II/III (Africa 2)	1	10
Type II/III	0	0
Atypique	0	0
TOTAL	10	100

with the QIAGEN ® Multiplex PCR kit (Qiagen, Courtaboeuf , France): 0.04 mM of each primer (Roche Diagnostics, Meylan, France), 4 and 6 mu.l DNA mu.l of water were added to the Multiplex Master Mix provided by QIAGEN (1x final concentration) in a total volume of 25 mu.l.

Gain realized by a thermocycler GeneAmp ® PCR System 2700 (Applied Biosystems, Courtaboeuf) was: initial denaturation at 95°C for 15 min; 35 cycles of amplification: 94°C (30 s), 63°C (3 min) and 72°C (60 s)/cycle; final extension at 60°C for 30 min. The PCR products were visualized with a molecular weight marker V (Roche Diagnostics, Meylan, France), after electrophoresis on an agarose gel (2% w/v) containing ethidium bromide (Figure 1). Depending on the intensity of the observed bands, the PCR products were diluted to 1/15 in the deionized formamide before electrophoresis by an automatic sequencer . 1 µl of each diluted product was mixed with 0.5 mu.l of size marker fluorescent ROX GeneScan ® 500 (75-500 bp, Applied Biosystems, Courtaboeuf) and 23 mu.l of deionized formamide. This mixture was denatured and analyzed by gel electrophoresis POP4 polyacrylamide held in a capillary (47cm/50 microns) (Applied Biosystems, Courtaboeuf). The fluorescence emission was recorded by an automated sequencer (310 Abiprism collection 1.0 , Applied Biosystems, Courtaboeuf) and analyzed by the GeneScan ® Analysis Software version 2.1 (Applied Biosystems, Courtaboeuf) .

Figure 2 shows the migration profile obtained for one isolate of type II after electrophoresis of PCR products on an automatic sequencer. Each significant peak fluorescence was an allele and its length was in bp. A genotype was characterized by a particular combination of alleles. An allele is often common to several types, which demonstrates the need for multilocus analysis to study the genetic diversity of Toxoplasma.Genotyping analysis Strain typing was performed by using the length polymorphism of 6 microsatellite markers in a modified multiplex assay. Elsewhere, we described a multiplex polymerase chain reaction (PCR) for typing strains of *T. gondii* by the use of five microsatellite markers (*TUB2, W35, TgM-A, B18* and *B17*). For this study, we added a sixth microsatellite marker to the multiplex assay *(M33)* which was located on chromosome IV.

RESULTS AND DISCUSSION

The study population consisted of 10 isolates from Dakar, addressed to CRB Toxoplasma to establish the genetic profile. It shows a predominance of genotype I/III or Africa 1 (90%) and I / II / III or Africa 2 (25%) (Table 1) as the lengths of their alleles were characterized using 15 microsatellite markers characteristics (Table 2).In our study, 10 from 10 isolates were recombinant type I / III or Africa 1 and one type I / II / III genotype or Africa 2, while isolates belonging to three clonally lines are minority (type III: 0/10, type II: 0/10 and type I:0/10). The corresponding isolates were isolated in France. These recombinant genotypes have typically a mixture of type I alleles with the alleles of type III. So, these few isolates indicate flow in Dakar (Senegal) recombinant I/III genotypes and I/II/III

Table 2. Multilocus microsatellite (MS) genotyping from 51 isolates of Toxoplasma gondii from manmade and wilderness in French Guiana (Ajzenberg et al 2010.)

Reference strains	Genotype MS															Genotype
	TUB2	**W35§**	**TgM-A**	**B18§**	**B17§**	**M33**	**MIV.1**	**MXI.1**	**M48**	**M102**	**N60**	**N82**	**AA**	**N61**	**N83**	
GT1	291	248 (1)	209	160 (1 ou 3)	342 (1)	169	274	358	209	168	145	119	265	087	306	Type I
ENT*	-	-	-	-	-	-	-	-	209	166	145	121	267	087	308	Type I
B1*	-	-	-	-	-	-	-	-	209	166	147	119	273	087	306	Type I
JONES*	289	242 (2)	207	158 (2)	336 (2 ou 3)	169	274	356	235	174	142	111	265	097	310	Type II
Me49	-	-	-	-	-	-	-	-	215	174	142	111	265	091	310	Type II
PRU*	-	-	-	-	-	-	-	-	209	176	142	117	265	121	310	Type II
CTG	289	242 (3)	205	160 (1 ou 3)	336 (2 ou 3)	165	278	356	215	190	147	111	269	089	312	Type III
NED*	-	-	-	-	-	-	-	-	209	190	147	111	267	091	312	Type III
VEG	-	-	-	-	-	-	-	-	213	188	153	111	267	089	312	Type III
GAB1-2007-FEL-CAT1	-	-	-	-	-	-	-	-	213	190	149	111	267	089	312	Type III
DPHT	291	248 (1)	205	160 (1 or 3)	336 (2 ou 3)	165	274	354	229	166	147	111	283	091	306	Africa 1
TgH 13002 (CCH-2004-NIA)*	289	248 (1)	205	160 (1 ou 3)	336 (2 ou 3)	165	274	354	225	166	145	111	273	089	306	Africa 2
GAB3-2007-GAL-DOM9	291	242 (3)	207	160 (1 ou 3)	342 (1)	165	274	354	229	166	142	111	273	095	310	Africa 3

Reference Strains: Type I (GT1, ENT and B1), Type II (JONES, Me49 and PRU), classic Type III (CTG, VEG and NED) Type III (GAB1-2007- 157 FEL-CAT1) and Africa 3 strain (GAB3-2007-GAL-DOM9) Recent native of Gabon and Africa 1 (DPHT) and Africa 2 (TgH 13002) strains isolated in this study. - ,Values identical to above; *, reference strain obtained at CRB Toxoplasma Limoges; §, The figures for conventional typing were adjusted after the sequencing of the W35, B17 and B18 markers; **( )**, Allelic polymorphism of these three markers are expressed as relative to conventional typing T. gondii: 1 alleles. **2, 3** are reserved for clonally lines I, II and III; allele 1 or 3 is that the type I and III share allele, allele 2 or 3 means that the Types II and III from the allele. The higher numbers correspond to unconventional alleles (atypical) and consider adjusting sequencing.

(Ajzenberg et al., 2004; Ajzenberg et al, 2009); designated respectively in terms of Africa 1 and Africa 2. It may be that the genotype Africa 1, which was mainly detected in immunocompromised patients in Africa West and Central is a clonally line (Ajzenberg et al., 2009). This genotype I / III have been isolated from chickens in Brazil or Portugal in cases of congenital toxoplasmosis. This said genotype "African" could represent a new clonally lineage, as we suggested in the first part. The fact of finding the same genotype I / III in several Brazilian isolates advocates this hypothesis (Pena et al., 2008; Ajzenberg, 2006). Similarly, Dubey and colleagues had described two recombinant strains Polish I / II / III (Dubey et al., 2008a). Aurelien Mercier described the genotype (African 1) in Gabon in 2010 and a new type Africa 3 for Africa. With regard to tropical Africa, Velmurugan et al. (2008) [Kenya, Nigeria, Congo, Mali, Burkina Faso and Ghana and Lindström et al. (2008) (Uganda) suggest that, as in Europe and the USA, Types I, II and III circulate with possible recombinant II / III (Lindström et al., 2009), although different genotypes have been reported earlier (Ajzenberg et al., 2009; Lehmann et al., 2006). So, it is important to remember that Ajzenberg technique allows better resolution in a single PCR and remains the most appropriate. Standardization of the technique could be surprises in these authors. These preliminary results are the only currently available, not necessarily trying to say that these are the only type recombinants circulating in Dakar because of the small sample, given the relatively high prevalence of the disease in women pregnant (45.81 ± 7.30% in women with 44.24 ± 7.58% in the pregnant) (Ndiaye, 2012, personal communication). It would be interesting to study in this direction on a more consistent sample taking into account the different clinical

forms to be affirmative.

Conclusion

Genotypes found in Dakar (Senegal) are recombinant genotypes mainly Africa 1 or Type I/III and Africa 2 or Type I/II/III. These two types are kept in CRB Toxoplasma at Limoges as reference strains. However, given the small size of the study population, it would be premature to say that those are the only strains circulating in the country. It would be interesting to make a more consistent sampling from all hosts involved to characterize the genetic polymorphism of *T. gondii* in Senegal. Also, making a correlation between genotypes and finding different clinical forms encountered would be of vital importance to our country

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