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Seroprevalence of *Toxoplasma gondii* and direct genotyping using minisequencing in free-range pigs in Burkina Faso

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ABSTRACT

Background: Swine are a major source of meat for humans. As such, they can play an important role in the epidemiology of human toxoplasmosis. Therefore, we performed an epidemiological study to determine the prevalence and genotypes of *Toxoplasma gondii* in Burkina Fasan swine.
Methods: The prevalence of *T. gondii* infection was evaluated in a 3-month prospective study at the slaughterhouse of Bobo-Dioulasso, Burkina Faso. Anti-*Toxoplasma* IgG titers were determined on meat juices from pig diaphragms using a commercially available ELISA assay. The DNA was extracted from 25 mg of heart biopsies of seropositive animals (IgG ≥ 50% of the control) and the presence of *T. gondii* DNA was detected using a quantitative PCR assay. Genotyping was performed directly on DNA from PCR-positive biopsies using high-resolution melting and minisequencing analyses of the repeated B1 gene.
Results: The prevalence of carcasses positive for anti-*Toxoplasma* IgG was 29% (87/300) with no difference according to sex and age in contrast to the village of origin ($p = 0.018$). Of the 87 seropositive animals, two were PCR positive (parasitic load at 64 and 128 parasites/mg of heart biopsy). Two new genotypes belonging to Type II and Type III and different from the genotypes previously described using minisequencing were identified.
Conclusion: Our study provides the first *T. gondii* seroprevalence data in Burkina Fasan swine. In addition, this direct typing method suggests diversity of the *T. gondii* genotypes circulating in domestic animals in Burkina Faso. This needs to be confirmed on a wider sampling of subjects.

1. Introduction

Human toxoplasmosis is due to the coccidian parasite *Toxoplasma gondii*. It is a food-borne disease transmitted by the ingestion of cysts contained in meat, or of oocysts on contaminated fruits and vegetables, or present in water. Toxoplasmosis is often asymptomatic but can lead to devastating diseases in immunocompromised individuals such as HIV positive patients, and fetuses. Pork is often considered to be lower risk than mutton for toxoplasmosis because it is often cooked more thoroughly (Dubey, 2009). This limits the risk of human transmission. However, in low resource countries, pork is the most consumed meat (http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html), and hogs can participate in local cycles involving other animals such as cats, rodents and chicken. Systems for routine diagnosis, monitoring or reporting of toxoplasmosis in pigs are often undeveloped (Dubey, 2009; Timbilfou et al., 2012). In addition, there is little information available on the epidemiology of toxoplasmosis. Thus, there is an urgent need to consolidate knowledge on toxoplasmosis—especially as the food supply becomes globalized with changes in culinary habits and increased international travel.

Genotyping of *T. gondii* has revealed genetic diversity and three main lineages (Types I, II, and III). These are now grouped into 15 haplogroups in six major clades (Su et al., 2012). Genotype determination is relevant for epidemiological reasons since genotypes circulating in Europe and North America are different from those identified in other part of the world, and also for clinical reasons (Xiao and Yolken, 2015). Indeed, pathogenicity in mice and in humans is different according to the genotype related to polymorphism in proteins such as dense granule and rhoptry (Melo et al., 2011). Severe inflammation in ocular toxoplasmosis was found associated with infection by strains harboring specific allele of the ROP18 gene (Sánchez et al., 2014).

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Several genotyping methods have been developed. The PCR-RFLP assay (for Restriction Fragment Length Polymorphism) is proposed as a global genotyping system but is limited by agar gel technologies with issues in reproducibility, lack of computerization for exchangeability (Su et al., 2006), and low sensitivity when dealing with low parasite DNA loads in tissue resulting in a variable proportion of genotyped samples (Bacci et al., 2015; Belfort-Neto et al., 2007; Wang et al., 2012). Microsatellite markers have emerged a very convenient means of genotyping since they were first reported (Costa et al., 1997). Nowadays, up to 13 markers in two multiplex assays are proposed for genotyping (Mercier et al., 2010). As with PCR-RFLP, the main technical limitation of the microsatellites markers is their low sensitivity. Because the targeted loci are only single copy, only high parasitic loads can be efficiently amplified and genotyped. To overcome this limitation, we developed a typing system based on the polymorphism of the repeated B1 gene of *T. gondii* using high-resolution melting (HRM) analysis and mini-sequencing (Costa et al., 2013, 2011).

Therefore, to determine the prevalence of *T. gondii* infection in pigs in Burkina Faso and to know the circulating strains, a prospective survey was performed at the Bobo-Dioulasso abattoir. Serology was performed on pork juices as previously reported (Hill et al., 2006). This was confirmed via real-time quantitative PCR (qPCR) for *T. gondii* DNA in heart biopsies of seropositive animals. The qPCR targeting the DNA repeated element of *T. gondii* has already been validated for human diagnosis (Reischl et al., 2003). Genotyping was performed on the qPCR-positive biopsies as previously reported (Costa et al., 2013, 2011).

2. Methods

2.1. Sampling protocol

Meat samples were collected between September 18th and December 12th 2008 at the slaughterhouse of Bobo-Dioulasso, the second largest city of Burkina Faso (813,610 inhabitants in 2014). The "Abattoir Frigorifique de Bobo-Dioulasso" (AFB) is the only authorized slaughterhouse of the city. According to a recent report, the pork meat produced at the AFB has increased 30% between 2001 and 2006 and represents 12% of the produced meat (Timbilfou et al., 2012). Most of the pigs (85%) come from surrounding villages whereas 15% come from intensive breeding in the city or in the close vicinity of the city (Timbilfou et al., 2012). Most of the production (89% of 672 tons) is consumed locally, mainly as baked pork, and 11% is exported (Timbilfou et al., 2012). The number of slaughtered pigs increases in November and December, the months chosen for our study (Timbilfou et al., 2012). During the study period, the veterinary meat inspection condemned 13 carcasses suspected of cysticercosis, which were not included in the study.

We therefore collected 300 pig samples. The age, gender and village of origin of each animal were recorded. For each pig, at least 100 g of the diaphragm as well as 50 g of the heart were collected in individual plastic bags, refrigerated at 4 °C and kept frozen at −20 °C after transportation to the laboratory. The meat juice was obtained from diaphragms cut into small pieces and frozen overnight at −20 °C in a plastic bag. After thawing at room temperature, the meat juice was centrifuged 15 min at 4000 rpm (1800*g*) and collected with a pipette into a microtube as previously described (Halos et al., 2010).

2.2. Detection of IgG

The ELISA test (ELISA ID Screen® Toxoplasmosis Indirect; ID VET, Montpellier, France) was performed on diaphragm fluids according to the manufacturer's instructions except that the dilution factor was 1:2 and not 1:10 due to weaker concentrations in body fluids versus sera (Halos et al., 2010). The positive control was *T. gondii*-positive polyclonal ovine serum diluted in a stabilizing solution. The threshold of positivity was a titer of anti-*Toxoplasma* IgG ≥50% of the control.

2.3. Detection of T. gondii DNA by real-time quantitative PCR (qPCR)

DNA was extracted from 25 mg of heart biopsy of seropositive pigs using the QIAmp DNA Mini Kit for tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Extracted DNA was eluted in 200 μl, and the DNA concentration was determined with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The 260 nm/280 nm and 260 nm/230 nm absorbance ratios were recorded with an expected value above 1.80 (range = 1.80–2.20). The qPCR targeting a repetitive DNA fragment (GenBank access number AF487550) in *T. gondii* was carried out in a StepOne® Instrument (Applied Biosystems).

Some modifications of the primers and probe were brought to our previous publication reporting Light-Cycler qPCR assays (Reischl et al., 2003) to take into account the present change for the Taqman format. The two primers (TG-CG10: 5′-ATCAGGACTGTAGATGAAGG CG-3′, and TG-CG11: 5′-TAGATCGCATTCCGGTGTCT-3′) and a hydrolysis probe (TG-CG12: 5′FAM-AGAAGATGTTTCCGGCTTGGCTGCTT-TAMRA3′) were designed to amplify and detect a conserved 140-bp region. In silico studies showed that the amplified region was specific for *T. gondii* when analyzed with the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/). No perfect alignment was observed with other organisms. One to five mismatches were present in primers and probes especially when compared to the closely related protozoan *Hammondia hammondi* (Genbank entries EU493285.1, EU493284.1, EU493282.1 EU493281.1, EU493283.1, EU493280.1 and EU493279.1). Such a repetitive element has not been described to date for *Neospora caninum*.

Serial dilutions of two reference strains of Type I (RH) and Type II (B7) with a known concentration of *T. gondii* tachyzoites were used to validate the qPCR assay. Standard curves were obtained using serial 10-fold dilutions in water of reference strain DNA from 2.10^5 to 20 tachyzoites per reaction. By plotting the quantitative cycle (Cq) against the input target quantity, we obtained the following figures: slope: −3.292; Y-intercept: 19.745; R^2: 0.98; and efficiency: 101.2%.

Five microliters of extracted DNA from heart biopsies was used per reaction. PCR was set up in a final volume of 20 μl with the Taqman Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France) containing uracil-*N*-glycosylase. Each primer and probe (Sigma, Paris, France) was used at 0.5 μM and 0.25 μM, respectively. The reaction mixture was initially incubated for 2 min at 50 °C followed by a 10-min step at 95 °C. Amplification was performed for 50 cycles of denaturation (95 °C for 15 s) and annealing/extension (65 °C for 1 min).

The results were expressed as the mean Cq of the duplicates tested for each specimen. Lower Cq values correspond to higher concentrations of the target DNA. During each run, a *T. gondii* DNA concentration corresponding to 100 *T. gondii* tachyzoites was used as a positive control (expected Cq = 33), and the elution buffer for DNA extraction was the negative control. The results were considered to be positive when a significant fluorescent signal above the baseline was detected. For 20 qPCR negative samples, the same *T. gondii* DNA concentration corresponding to 100 of *T. gondii* tachyzoites was used for detection of PCR inhibitors. After adding *T. gondii* DNA to the DNAs extracted from these 20 qPCR negative samples, the amplification was performed as above.

2.4. T. gondii genotyping

Genotyping of *T. gondii* was based on an analysis of eight nucleotide polymorphisms (SNPs) located within the B1 gene as described elsewhere (Costa et al., 2013, 2011). Briefly, 8 SNPs were studied with the HRM analysis of PCR products followed by purification of the PCR products and minisequencing analysis using the SNaPshot Multiplex kit (Applied Biosystems, Courtaboeuf, France). The reactions were run on an ABI3130XL genetic analyzer and analyzed using the Genescan software. DNA from reference strains for each of the three main lineages—Type I (RH strain), Type II (B7 strain), and Type III (C5 strain)—were kindly