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 titer 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 pork. 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.

Article info

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 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).
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 (85% 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 (Ttimbilfou 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 (1800g) 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 QIAamp 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 LightCycler qPCR assays (Reischl et al., 2003) to take into account the present change for the Taqman format. The two primers (TG-CG10: 5′–ATCAGGACTGTAGATAAGGGCG-3′, and TG-CG11: 5′–TAGATCCGATTCCGGTGCT-3′) and a hydrolysis probe (TG–CG12: 5′-FAM-AGAAGATGTCTCCCGCGTCTTTATMRA3′) 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 hammondii (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 to 20 tachyzoites per reaction. By plotting the quantitative cycle (Cq) against the input target quantity, we obtained the following figures: slope: −3.292; R2: 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 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 (CS strain)—were kindly
provided by Asis Khan (David Sibley laboratory). Strains from Africa (Africa 1 (RMS-2003-DJO, thereafter DJO) and Africa 2 (CCH-2004-NIA, thereafter NIA), and the Caribbean (CCH-2005-REN, thereafter REN) were purchased from the French National Center of Reference for toxoplasmosis.

The genotypes of the qPCR-positive animals were therefore determined together with six reference strains (RH, B7, C5, DJO, NIA, and REN). Twenty-three representative genotypes have already been obtained from amniotic fluids (Costa et al., 2013).

2.5. Statistical analysis

Data are presented as the mean ± standard error or median and range. Statistical analyses were performed using Prism v4.0 (GraphPAD Software, San Diego, CA). A value of p < 0.05 was considered to be statistically significant.

3. Results

Among the 300 pig carcasses (mean age: 4 months; range 1–37) studied, anti-Toxoplasma seropositivity was 29% (87/300) (Table 1). There was no significant difference between the prevalence in boars and sows, 25.3% (39/154) and 32.9% (48/146), respectively, or between the age and titers of anti-T. gondii IgG ($r^2 = 0.002$). Considering the 244 animals for which the village of origin was known, there was a significant difference ($p = 0.018$) according to the geographical distribution of the seropositive (anti-Toxoplasma IgG ≥ 50%) animals (Table 1).

The heart biopsies of the 87 seropositive animals were tested for the presence of T. gondii DNA. The DNA concentrations of the pig heart biopsies were 201 ± 96 ng/μl with a 260/208 ratio of 1.9 ± 0.05. No amplification was seen in the negative controls. To test for PCR inhibitors, 20 samples were added to the same T. gondii DNA quantity as the positive control. The Cq values were 33.5 ± 0.25, which is similar to the ones obtained with the positive control. This demonstrates the absence of PCR inhibitors. In these conditions, two animals were positive via qPCR (Pig 1: a 37-month old male of unknown origin; and Pig 2: a 3-month old male from Dandé village). The Cqs were 33 and 32, which corresponded to 64 and 128 parasites/mg in the heart biopsy, respectively.

To place the positive animals in a hierarchical clustering, the genotypes obtained for the two qPCR positive animals were analyzed with the genotypes of 7 reference strains and 23 congenital infection strains (Costa et al., 2013). Our clustering was already shown to fit with the three major lineages (Types I, II, and III). Using the arbitrary threshold of 1.25, two clades were observed in lineages II (IIa, and IIb). One qPCR-positive animal (Pig 1) clustered in Clade IIb, along with one from French Guiana and one from La Réunion Island amniotic fluids (Costa et al., 2013). None of the two pig genotypes demonstrated the absence of PCR inhibitors. In these conditions, two animals were positive via qPCR (Pig 1: a 37-month old male of unknown origin; and Pig 2: a 3-month old male from Dandé village). The Cqs were 33 and 32, which corresponded to 64 and 128 parasites/mg in the heart biopsy, respectively.

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4. Discussion

This is the first serological study in pigs from different villages slaughtered at Bobo Dioulasso, Burkina Faso. Our results show a 29% (87/300) seropositivity of IgG specific for Toxoplasma. We used an ELISA kit licensed by ID VET, France, to detect T. gondii antibodies in pigs. This ELISA kit is not specific for a given animal species; it has already been used for epidemiological studies of many species including pigs and wild boars (Arko-Mensah et al., 2000; Halos et al., 2010; Opsteegh et al., 2010b; Prangé et al., 2009; Richomme et al., 2010). However, interpretation and generalization of the results should be cautious. Indeed, there is no general agreement among the techniques and

<p>| Age, sex ratio, and anti-Toxoplasma IgG ≥ 50% according to the geographical origin of the 300 pig carcasses studied |
|---|---|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Geographical origin (village or city)</th>
<th>n (%)</th>
<th>Median age (mo) (range)</th>
<th>Anti-Toxoplasma IgG ≥ 50% n (%)</th>
<th>Anti-Toxoplasma IgG ≥ 50% p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bobo-Dioulasso</td>
<td>32 (10.7)</td>
<td>3 (2–5)</td>
<td>8 (25)</td>
<td>0.001</td>
</tr>
<tr>
<td>Dandé</td>
<td>12 (3.7)</td>
<td>2 (1–4)</td>
<td>4 (12)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Douna</td>
<td>19 (6.3)</td>
<td>1 (1)</td>
<td>6 (18.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Faramanan</td>
<td>17 (5.7)</td>
<td>1 (1)</td>
<td>5 (15)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Kouroma</td>
<td>10 (3.3)</td>
<td>1 (1)</td>
<td>3 (10)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ndorola</td>
<td>14 (4.7)</td>
<td>1 (1)</td>
<td>4 (14)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Niangelogo</td>
<td>19 (6.3)</td>
<td>1 (1)</td>
<td>6 (18.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Samoroga</td>
<td>8 (2.6)</td>
<td>1 (1)</td>
<td>2 (6.3)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (1.7)</td>
<td>1 (1)</td>
<td>1 (3)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>4 (1–4)</td>
<td>87 (29)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* For the 244 animals for which the origin is known.
cut-off, no standardized reference materials, and no quality control program (Dubey, 2009). In addition, discrepancies can also rely on storage conditions, hemolysis, microbial contamination, or repeated freezing-thawing cycles.

Our second technical choice was to use muscle fluids instead of serum samples. Using the same *T. gondii* ELISA assay, tissue fluids were found to be considerably less efficient than serum samples (Gamble et al., 2005). Therefore, our estimate for *Toxoplasma* seroprevalence is likely to be lower than if serum were used. Another limitation is the non-validation of the test in developing countries where multiparasitism is common. Although not specifically studied here, we highlight the high number of cysticercosis cases (*n* = 13 at the slaughterhouse of Bobo-Dioulasso). This is another major parasitic disease transmitted via pork in Burkina Faso (Carabin et al., 2015). Similarly, *Sarcocystis* spp. infections could be responsible for cross reactivity with *T. gondii* antigens (Damriyasa et al., 2004). However, this is a pitfall of every study dealing with free-range pigs in tropical countries. Nevertheless, we used a commercially available assay to assume a certain reproducibility and availability.

*T. gondii* is rarely detected in confinement raised swine from developed countries (Dubey, 2009) although the development of organic farms can modify this general statement (Bacci et al., 2015; Dubey et al., 2012). In sub-Saharan countries and more specifically in Burkina Faso, pigs are mainly raised in a traditional manner in small farming communities (Carabin et al., 2015; Timbilifu et al., 2012). Except for one study from Côte d’Ivoire (Prangé et al., 2009), that reported a seroprevalence in pork samples of 8.8% [range, 8.2–9.37], the prevalence was usually high, including 20–36% in Zimbabwe (Hove et al., 2005), 29% in Nigeria (Onyiche and Ademola, 2015), 32% in Ethiopia (Gebremedhin et al., 2015), and 39% in Ghana (Arkomensah et al., 2000). The present seroprevalence (29%) is similar confirming the importance of pigs as intermediate hosts of *T. gondii*. The high positivity rate is explained by traditional breeding with occasional backyard scavenging pigs (Arkomensah et al., 2000; Hove et al., 2005; Prangé et al., 2009). Interestingly, differences in seroprevalence in the present study are more linked to a specific village than to gender or age as previously suggested for *Toxoplasma* in Ghana (Arkomensah et al., 2000), suggesting very location-specific epidemiology.

For the detection of *T. gondii* DNA, we studied heart tissue because this is one of the most commonly infected sites (Esteban-Redondo and Innes, 1998). Only two heart biopsies of the 87 were qPCR positive. The detection of *T. gondii* tissue cysts in organs of large animals is difficult due to their heterogeneous distribution. Thus, little is known about tissue cyst density. Indeed, the commercial DNA extraction kit is designed for 25 mg of tissue. This cannot assume that *T. gondii* cysts were present in the processed biopsies. As a consequence, the most sensitive method for *T. gondii* detection in meat products is still the demonstration of the presence of the parasite by mouse bioassays because 50 g of meat can be processed (Dubey et al., 2012), whereas the quantity of meat amenable to DNA extraction is much lower (25 mg in the present study using a commercial kit). However, the higher sensitivity of bioassays could be challenged when using newer molecular tools with an initial DNA enrichment (Gomez-Samblas et al., 2015; Opsteegh et al., 2010a).

The present minisequencing method was developed to genotype even in the presence of low amounts of parasite DNA (Costa et al.,...
between age and this can explain, at least in part, the lack of correlation between swine reported here echoes what is seen in humans. Indeed, whatever ranging pigs live adjacent with human beings and are a sentinel of infections. The number of genetic markers (n = 8) is lower than the system based on 13 microsatellite markers (Mercier et al., 2010). The SNPs are one locus can introduce bias usually resulting in an underestimation of genetic differences. Therefore, a strict comparison with the genotypes reported to be more virulent in mice (Mercier et al., 2010). The minisequencing method, nevertheless, presents some limitations. The number of genetic markers (n = 8) is lower than the system based on 13 microsatellite markers (Mercier et al., 2010). The SNPs are also all in the same locus, the B1 gene—in contrast to the microsatellites markers which are more evenly spread across the genome. Studying one locus can introduce bias usually resulting in an underestimation of genetic differences. Therefore, a strict comparison with the genotypes reported in Africa using microsatellite markers (Mercier et al., 2010) was not possible. The main advantage of our system over microsatellite markers is the avoidance of a bioassay designed to amplify live parasites by inoculation in mice. This bioassay introduces bias when all animals from a given species are not all positive (Dubey et al., 2012; Mercier et al., 2010) or when T. gondii from a given intermediate host are not viable in mice (Aroussi et al., 2015). To understand the role of free-range pigs in the epidemiology of local toxoplasmosis and more specifically for human infection, more information is needed about the genotypes infecting the local populations. The seropositivity of animals strongly correlates with live, infective tissue cysts in meat (Dubey, 2009). Therefore, seropositive pork could be the source of human infection either directly, although this meat is either not consumed for religious beliefs or always cooked by feral cats in the vicinity of the slaughterhouse. The other method of human infection is indeed the ingestion of oocysts excreted by cats and the role of other intermediate hosts cannot be excluded. Poor sanitation could be a cause of contamination of the food by oocysts although oocysts are known to be rapidly killed at temperatures >45 °C (Dubey, 1998)—this is often reached in summer months in Burkina Faso. Incidentally, if ingestion of environmental oocysts does not occur during the summer, the pigs are not progressively infected with increasing age and this can explain, at least in part, the lack of correlation between Toxoplasma seroprevalence in pigs and age. In addition, free-ranging pigs live adjacent with human beings and are a sentinel of infection for the human population. The high prevalence of infection in swine reported here echoes what is seen in humans. Indeed, whatever the source of human infection, the Toxoplasma-seroprevalence in pregnant women is 33.1% at Bobo-Dioulasso. This suggests that maternal transmission to the fetus is possible (Bamba et al., 2014).

5. Conclusion

The high rate of Toxoplasma seropositivity in Burkina Faso free-range pigs highlights the need to monitor the spread of the parasite and the need to reduce its transmission. In addition, our study revealed variability of the genotypes circulating in small geographic areas, underlining the need for further genotype studies for epidemiological purposes. Genotyping of human cases in Burkina should help elucidate the source of infection.

References


