Occurrence of Toxoplasma gondii in Carcasses of Pigs Reared in Intensive Systems in Northern Italy

ROBERTO PAPINI,1 PIERLUIGI DI CICCIO,2 MARIANNA MARANGI,3 SERGIO GHIDINI,2 EMANUELA ZANARDI,2

ALBERTO VERGARA,4 ANNUNZIATA GIANGASPERO,3 SIMONA NARDONI,1 GUIDO ROCCHIGIANI,1

FRANCESCA MANCIANTI,1 AND ADRIANA IANIERI2*

1Dipartimento di Scienze Veterinarie, Universita degli Studi di Pisa, Viale delle Piagge 2, 56124 Pisa, Italy; ` 2Dipartimento di Scienze degli Alimenti,

Universita degli Studi di Parma, Via del Taglio 10, 43126 Parma, Italy; ` 3Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Universita degli `

Studi di Foggia, Via Napoli 25, 71121 Foggia, Italy; and 4Facolta di Medicina Veterinaria, Universit ` a degli Studi di Teramo, SP 18, 64100 Teramo, Italy `

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ABSTRACT

To evaluate the occurrence of Toxoplasma gondii and to genetically characterize its isolates in carcasses of industrial fattening pigs, blood, diaphragm, and heart samples were collected from 375 carcasses of pigs slaughtered to be processed for Parma ham production. Pigs had been bred on approved farms (n ¼ 75) located in the so-called Food Valley in Italy. Sera were examined for immunoglobulin G antibodies to T. gondii by modified agglutination test (MAT). Both heart and diaphragm samples from seropositive carcasses were processed for the presence of T. gondii DNA (B1 locus) by real-time PCR and high resolution melting (HRM) assay. Anti-Toxoplasma antibodies were detected in 2.1% of pig carcasses, with titers from 1:10 to 1:320. T. gondii DNA was detected in all (eight) seropositive carcasses and in 11 (5 heart and 6 diaphragm samples) of 16 samples; that is, it was detected in heart tissue in two subjects, in diaphragm tissue in three subjects, and in both muscle tissues in three subjects. Toxoplasma genotypes were determined in seven of eight carcasses: type III was identified in four carcasses, type II in two, and both III and II in one carcass. The serological findings and the molecular detection of T. gondii strains suggest that cured meat products obtained from industrially bred pigs may be potential sources of toxoplasmosis for humans. Our results provide novel, important information regarding the seroprevalence and molecular prevalence of T. gondii in intensively reared pigs within this specific region of Italy, particularly because Parma ham from this region is known and consumed worldwide. Onfarm preventive measures combined with slaughterhouse monitoring of carcasses of pigs bred for cured meat production should never be overlooked to prevent the introduction of T. gondii into the food chain and to ensure safety for consumers of these products.

Key words: High resolution melting assay; Industrial swine; Lineages; Real-time PCR; Serology; Toxoplasma gondii

The cosmopolitan parasite Toxoplasma gondii (Apicomplexa, Coccidia) is an obligate intracellular protist and is the etiological agent of toxoplasmosis. Cats and other felids act as definitive hosts, shedding unsporulated oocysts of T. gondii with their feces in the environment (13), whereas all other warm-blooded animals may serve as intermediate hosts. Humans and domestic and wild animals may acquire toxoplasmosis by the consumption of raw or undercooked meat containing encysted bradyzoites and/or by ingestion of sporulated, environmentally resistant oocysts contaminating soil, food, or water. Transplacental transmission may also occur, mostly in humans and sheep (3, 11, 13, 24). Primary infection is usually subclinical in immunocompetent individuals (i.e., animals and humans). Toxoplasmosis is of

considerable concern for public health because of the risk of stillbirth and congenital malformations in serologically negative pregnant women (3). Moreover, reactivation of latent infection can occur in immunocompromised patients and may cause life-threatening encephalitis, although the prognosis for HIV patients has improved considerably since the advent of highly active antiretroviral therapy (6). Three predominant T. gondii clonal lineages have been identified and are designated as type I, II, or III; it has been reported that they are widespread in North America and Europe and that they show different virulence in mice (22, 34). However, other studies carried out in recent years indicate either that T. gondii type I is very rare or that it has a restricted host range in Europe (1, 19). Other genotypes and atypical strains, which do not fit into these three major lineages, have also been identified but are rare in Europe (33). Among meat-producing animals, pigs are considered one of the most important sources for human infection (11). Swine breeding is important for the economy of many countries; this is particularly true in Italy, which is known worldwide for pork products of the highest quality. This pork is labeled as Protected Designation of Origin (PDO) or Protected Geographical Indication (8). Several serologic surveys on the prevalence of T. gondii in pigs from different breeding systems have been performed in Europe (11), including Italy, and have found rates of 10.4% (38), 16.14% (37), 9 to 64.4% (32), and 95.2% (2). However, there are limited data related to genetic characterization of T. gondii in pigs in this country; a recent report of type I, II, and I-II patterns (2) focused on outdoor bred animals. T. gondii seroprevalence in industrially kept fattening pigs has continued to drop dramatically over the years due to major changes made in pig production and management systems (3, 11, 13, 15). In most industrialized countries, seroprevalence in slaughtered pigs is now ,5% (13). A nationwide survey of T. gondii in meat (chicken, beef, and pork) obtained from retail stores in the United States found a seroprevalence of only 0.57% in pork (12). Although data from a number of different countries show that seroprevalence of Toxoplasma among pigs has fallen, periodic surveillance and monitoring for T. gondii infection is highly recommended in pigs raised in intensive farming systems as well as in other livestock species (15). Thus, the aim of this surveillance study was to estimate the occurrence of T. gondii in carcasses of pigs (Large White, Landrace, Duroc, or their hybrids) with a minimum weight of 150 kilos, bred and slaughtered in the North of Italy to be used in PDO products, and to genetically characterize the isolates by realtime PCR and high resolution melting (HRM) assay.

MATERIALS AND METHODS

Study area, farms, and animals.

The investigated area in Italy is known for the production of special pork products and covers a territory with the largest production of PDO Parma ham, i.e., the so-called Food Valley (which extends through the Piedmont, Lombardy, Emilia-Romagna, and Veneto regions along the Po River), where more than 90% of authorized farms that breed pigs used for the production of Parma ham are located. To qualify for Parma ham production, pigs must be born and raised according to mandatory requirements and special conditions on approved farms in 1 of 10 regions of northern and central Italy. Only Large White, Landrace, and Duroc breeds of pigs, or their hybrids, can be used. The diet of pigs to be processed for Parma ham is tightly controlled and specially regulated; it includes a specially formulated blend of grains, cereals, and whey from locally made Parmigiano-Reggiano cheese production. The pigs must weigh around 160 kg and be more than 9 months old at slaughter, and they must be slaughtered in a defined area (http://www.prosciuttodiparma.com/pdf/en_UK/Specifications.pdf). Effective control measures against pests (rodents, flies, cockroaches, and other insects) are commonly applied as an integral part of maintaining high health and productivity levels. Between January and June 2015, 375 pig carcasses were examined. Pigs were slaughtered in an abattoir located in the province of Parma (latitude: 448480 N, longitude: 108200 E), Northern Italy. Only pigs (about 400/h) were slaughtered at this facility. All slaughtered pigs were certified to be processed for PDO Parma ham production. A batch was defined as a group of 120 pig carcasses from a single farm on a given day. Each batch examined (for a total of 75 batches) came from one farm (for a total of 75 farms). Of each batch, five pig carcasses were randomly selected from the conveyor belt for

bleeding, with an interval of at least 10 animals between batches. The sample size (n ¼ 375) was calculated to be representative of 75 batches (accounting for a total number of 9,000 carcasses). Sampled carcasses were identified by date, farm code, and area of origin, and they were followed along the slaughter line for sampling.

Sample collection.

Immediately after shackling and hoisting, blood samples were individually collected from the jugular vein of 375 subjects into 50-mL falcon vials, labeled for pig carcass identification, and allowed to clot at room temperature. Heart and diaphragm samples (approximately 25 g each) were also collected from the same carcasses during the postmortem inspection, labeled as above, and put into individual plastic bags. Serum and muscular tissue samples were placed in an insulated container packed with ice or cold packs and were brought to the laboratory. The sera were transferred in Eppendorf tubes after 15 min of centrifugation at 2,000 3 g and were stored at 208C until serological analysis was performed. Muscular tissue samples were stored in turn at 208C pending molecular analyses.

Serological analysis.

Serum samples were examined for anti– T. gondii immunoglobulin G antibodies by the modified agglutination test (MAT). A commercial kit (Toxo Screen DA, bioMerieux, Lyon, France) with formalin-treated ´ Toxoplasma tachyzoites as antigen was used according to the manufacturer's instructions. Positive and negative control sera were included in each test. Serum samples were diluted twofold, starting at a 1:10 dilution until the end-point titer was reached. Sera with MAT titers of 1:10 or higher were considered positive. The sensitivity and specificity of the test were assumed to be 85.7 and 94.6%, respectively, as previously determined by a comparison study with bioassay in cats (16). Isolation of genomic DNA. A total of 16 heart and diaphragm samples from all seropositive subjects (n ¼ 8) were thawed and individually homogenized in 0.04 M phosphatebuffered saline (pH 7.2 to 7.4). Then, 25-mg aliquots of matrix were subjected to three freeze-thaw cycles (808C and then þ808C for 5 min) followed by DNA extraction, using the Tissue Genomic DNA Extraction Kit (Fisher Molecular Biology, Trevose, PA) according to the manufacturer's instructions. Samples from the heart and diaphragm of seronegative carcasses were discarded.

Real-time PCR performance.

The analytical sensitivity of the assay was estimated using 10-fold serial dilutions (from 1010 to 10 copies per lL) of the one reference strain that was subjected (in triplicate) to real-time PCR and subsequent melting curve analysis, and the mean value of the threshold cycle (CT) was plotted against the logarithm of DNA copies per lL. The standard curve was produced by a linear regression of the plotted points, and the range of linearity and the lowest detectable amount of DNA were estimated. PCR efficiency (E) was calculated according to this equation: E ¼ 101 /slope 1 (31). Values considered acceptable were E between 90 and 110% and correlation (R2) ,1. To determine repeatability, three standard points (107 , 105 , and 10 copies per lL) were tested in triplicate three times in the same experiment, and the CT mean values were registered. The same standards were tested once a day for three more days to determine reproducibility. To analyze the reproducibility and repeatability, both intra- and interassay coefficients of variation (CV) mean values were assessed. CV mean values were calculated following this formula: CV¼r(CT)/l(CT), where r is the sum of CT and l is the number of CT.

Real-time PCR and HRM assay.

The real-time PCR and HRM assay were performed in a CFX-96 Real Time Instrument (Bio-Rad, Segrate, Milan, Italy) (27). Briefly, PCRs were carried out in a final volume of 20 lL, utilizing SsoFast EvaGreen Supermix (cat. no. 172-5201, Bio-Rad) and 0.5 lM each speciesspecific primer ToxB41f (forward: 50 -

TCGAAGCTGAGATGCTCAAAGTC-30) and ToxB169r (reverse: 50 -AATCCACGTCTGGGAAGAACTC-30) capable to amplify a region of about 129 bp within the 35-fold repetitive B1 locus of T. gondii (AF179871) (5). Genomic DNA, positive controls (0.5 pg; reference strains for types I, II, and III), and negative control (ultrapure Milli-Q water, Millipore, Billerica, MA) in 5 lL were added to the reaction. PCR cycling conditions were as follows: initial denaturation at 988C for 2 min, followed by 40 cycles at 988C for 5 s, and 628C for 15 s. Fluorescence data were collected at the end of each cycle as a single acquisition. To determine the lineages of T. gondii samples, HRM assay was performed at the end of each PCR run (70 to 958C at 0.58C/5 s) using Precision Melt Analysis software version 1.2 (Bio-Rad). T. gondii B1 plasmid types I, II, and III and each sample were run in duplicate, and the amplification CT and melting temperature (Tm) mean values were calculated. The criteria used to define a test-positive sample were (i) a detectable amplification curve, (ii) a Tm value equal to the Tm value of one of the three reference strains, and (iii) a rate of change in fluorescence (dF/dT) value of .2. In the first step of HRM analysis, raw data were normalized by applying curve scaling to a line of best fit, so that the highest fluorescence value was equal to 100 and the lowest was equal to zero (a standard normalized melt curve). Next, the curves were differentiated, and a composite median curve was constructed using the median fluorescence of all samples. The melt traces for each sample were subtracted from this composite median curve to draw a residual plot (a difference graph). Here, differences in melting curve shapes and Tm points were used to genotype the samples. The difference plot obtained analyzes the differences in melting curve shape by subtracting the curves from each plasmid type I, II, III and DNA samples; this makes it possible to cluster the samples into groups according to nucleotide variations present in the sequences for plasmid types I, II, and III.

Sequencing.

Real-time PCR samples positive to T. gondii were purified using EXO I and FAST AP enzymes (Fisher Scientific, Landsmeer, The Netherlands) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions with the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), using the same primers as the respective PCR reactions according to the manufacturer's instructions. An ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) was used to determine sequences, electropherograms were inspected by eye, and consensus sequences were determined. Each sequence was compared with the nucleotide sequences available in publicly accessible databases using the National Center for Biotechnology Information's BLASTn software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, sequences were aligned using the ClustalW program in BioEdit software version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and each sequence was analyzed at the two diagnostic nucleotide positions (nucleotide no. 94 and 114) according to Costa et al. (7) to confirm type I, II and/or III clonal lineages identified in the HRM assay.

Statistical analysis.

The proportion of Toxoplasma-positive carcasses was calculated as number of positive carcasses , number of examined carcasses 3 100 along with the corresponding exact binomial confidence interval for proportions. The true prevalence of T. gondii infection was determined as follows: [apparent prevalence þ (test specificity 1)] , [test sensitivity þ (test specificity 1)].

RESULTS

Serological results.

Anti-Toxoplasma antibodies were detected in 8 (2.1%, exact binomial confidence interval: 0.9 to 4.2%) of 375 pig carcasses, with titers ranging between 1:10 and 1:320 (Table 1). Assuming an apparent prevalence of 0.0213, with specificity and sensitivity values of 94.6 and 85.7% for MAT in pigs, respectively (16), an estimate of the true prevalence was calculated as follows: [0.0213 þ (0.946 1)] , [0.857 þ (0.946 1)] ¼ –

0.0327/0.803 ¼0.0407. Because we ended up with a negative value, the true prevalence was ,0. Therefore, it was not possible to assess the true prevalence of T. gondii infection in the swine population examined.

Molecular results.

Upon assessing assay performance, we established that intra-assay CV values for the 107 , 105 , and 10 standard points were 1.7, 1.65, and 1.3% and that interassay CV values for the 107 , 105 , and 10 standard points were 0.85, 0.62, and 0.91%. The range of linearity was acceptable from 1010 to 10 copies per lL, with an amplification E of 103.1% and a slope of 3,251 (R2 ¼ 0.992). Differences in the melting curves, in the melting peaks, and in the Tm for plasmid types I, II, and III analyzed by real-time PCR assay are shown in Figure 1. T. gondii DNA was detected in all eight seropositive animals subjected to molecular analysis and in 11 of 16 muscle samples molecularly examined from those subjects, including five of eight cardiac muscle samples and six of eight diaphragm samples. Among the eight seropositive pigs, two pigs harbored T. gondii DNA in their cardiac muscles alone, three in their diaphragms alone, and three in both of these muscle samples. T. gondii–positive samples and reference strains analyzed by real-time PCR assay, as expected, showed overlapping peaks (data not shown) due to the very similar Tm values, whereas HRM analysis demonstrated that the samples clustered in two groups according to nucleotide variation, in particular three samples clustered in type II group and seven samples clustered in type III group (Fig. 2).

Among the pigs, types II and III were identified in two and four individuals, respectively, whereas in one subject, type II in heart and type III in diaphragm were detected (Table 1). In one diaphragm sample positive for T. gondii DNA, the melting curve was not well defined, and thus type identification in one serologically positive pig was challenging. Sequencing showed that three samples belonged to type II and seven samples belonged to type III (Fig. 3), confirming the results obtained in the HRM assay.

DISCUSSION

The origin of the infection on the investigated farms can only be hypothesized: industrially reared pigs might have acquired the infection through accidental ingestion of infected rodents and birds or other environmental pests, such as cockroaches or flies, whose potential role in mechanical transmission of Toxoplasma is well documented (18, 26). There are not official programs in place to monitor the parasite in pigs on farms and at slaughter. Possible methods for testing slaughtered animals include serology and bioassay, i.e., feeding cats or mice with meat from animals infected with viable T. gondii irrespective of their serological status and then searching for oocysts in cat feces or tissue cysts in mouse brain compatible with T. gondii infection. Although cat bioassay is considered to be the ‘‘gold standard’’ for the detection of T. gondii infection, its routine application is not feasible because it is costly and time-consuming and requires strict ethical procedures; conversely, serology is much more practical for routine analyses and is regarded as a good indicator of the presence of Toxoplasma in pig meat (13). Moreover, MAT showed a much higher sensitivity for detection of T. gondii in naturally and experimentally infected swine when compared to molecular tools, using mouse bioassay as a reference test (20). Seropositivity to T. gondii in pigs has been investigated worldwide. In Europe, prevalence varies widely, from 0% in fattening pigs raised in confinement in The Netherlands (23, 36) and Romania (29) to 95.2% recently registered in organic free-ranging pigs in Italy (2). In this country, seroprevalence values ranging from 9 to 64.4% were also reported among different populations of indoor-farmed pigs (32, 37, 38). In the present study, in which carcasses from industrially kept fattening pigs were exclusively examined at the abattoir, a considerably lower (2.1%) infection rate was found, compared with the previous reports. This value is in agreement with the results of other studies carried out in pigs intensively reared in other European countries, i.e., the Czech Republic, Romania, and Slovakia, where seroprevalence values between 0.4 and 2.06% were detected (21, 35, 39). There is evidence that seropositivity rates may vary greatly, depending on different diagnostic tests, cut-off values, countries, and geographical areas within the same country; in this study, MAT was chosen as the serological assay because it is still considered to be the most sensitive and specific test for the

serodiagnosis of toxoplasmosis in swine (14, 16). As to Toxoplasma lineages, clonal types I, II, and III strains show different virulence in mouse models, with type I strains being uniformly lethal for mice, irrespective of the dose, and types II and III being less pathogenic (11, 13). In our study, results of real-time PCR and HRM assay showed that pigs had been exposed to infection with clonal T. gondii lineages II and III. This is in agreement with previous findings in 15 isolates from Portugal, where clonal lineages II and III were found circulating in pigs (10), and also partially overlaps the very recent data obtained by Bacci et al. (2), who detected types II or I and II in organic pigs in Italy. In our survey, note that one of eight seropositive pigs was infected by two genetically distinct strains of T. gondii. Considering the relatively small geographic area, the indoor intensive farming system with pigs reared in close confinement, and the low prevalence of T. gondii infection found in pigs, it is unknown how one pig would have been exposed to coinfections with more than one parasite genotype during its lifetime. About 50 years ago, pork was considered to be an important source of Toxoplasma infection for humans (3, 11, 13, 24), and the risk was related to swine breeding management. In swine bred in modern industrial and intensive farming systems, the prevalence of Toxoplasma has drastically dropped due to management measures, i.e., confinement rearing on concrete floors, systematic rodent control, hygienic feed handling procedures, exclusion of cats, and other biosecurity systems (3, 11, 13, 24). Exhaustive reviews in specialist literature point out a .90% decline in Toxoplasma prevalence in commercial market pigs over the last two decades, and T. gondii infection has been virtually eliminated in pigs reared in current modern production systems (3, 9, 11, 13, 15, 24). Currently, the risk of acquiring toxoplasmosis from swine products is increased only by consumption of raw or undercooked pork from free-ranging pigs reared outdoors in organic systems (11). Therefore, this study confirms that, in intensive swine farms, the risk of exposure to T. gondii for the animals and, consequently, for the general human population, may be considered low; however, it cannot be underestimated. The viability of Toxoplasma tissue cysts can be negatively affected by the postslaughter treatments to which swine products are subjected, including salting, resting, and ripening phases, curing, storage, and hightemperature cooking (more than 608C) (3, 11, 25). The survival of Toxoplasma tissue cysts (and, consequently, the safety of dry cured ham) depends on the manufacturing process carried out for ham production, i.e., curing time, salt, nitrite concentration, etc., which may vary according to the local, regional, and national parameters used to manufacture ham. The viability of T. gondii in hams from pigs experimentally infected with T. gondii oocysts and subjected to all the typical procedures for PDO Parma ham production has recently been studied (17). In this study, both molecular and bioassay tests provided evidence that T. gondii infectivity was eliminated, confirming that the manufacturing process seems to inactivate Toxoplasma tissue cysts in Italian Parma ham (17). In conclusion, this is a comprehensive serological and molecular study of the occurrence of Toxoplasma in carcasses of industrially kept fattening pigs reared in an economically important area of Italy. Undoubtedly, intensive farming is not conducive to a high exposure level to T. gondii in swine. However, the serological (8 seropositive of 375 tested) and molecular (no type I) results of the present survey suggest that meat products from pigs intensively reared in the so-called Italian Food Valley and mainly bred for ham production may play a role as potential sources of toxoplasmosis for humans. Unfortunately, no bioassay was done to determine the safety of meat from the examined pig carcasses because this was beyond the aims of the present study. Nonetheless, note that cured meats available in the marketplace vary in composition and curing time and that the efficiency of the curing process for the inactivation of T. gondii may be questioned, because NaCl levels may not achieve 3%, which is necessary to kill cysts in many PDO products that are aged for a short time (30); this, however, does not seem to be the case for Parma ham (17). A potential risk (even though limited) still remains related to the consumption of undercooked or raw meat from those pigs. In The Netherlands, the results of a quantitative risk assessment for meatborne T. gondii infection demonstrated that, even with low prevalence of infection in cattle, consumption of beef remained an important source of infection (28). An epidemiological study of risk factors for recent Toxoplasma infection in pregnant women in Southern Italy found a strong association with eating cured pork (4). Viable T. gondii was detected in 1 of 67 ready-to-eat cured meat samples, indicating a failure of the commercial curing process (40). All these data from the

literature imply that the risk of toxoplasmosis from meat of industrially reared pigs might be very low for the average population of consumers; however, even a few human cases might have a great impact on consumer purchasing behavior. Indeed, based on their perceptions, consumers are keen to link the risk of human toxoplasmosis to preparation and ingestion of pork and other meats, and this attitude might negatively affect the demand for pork products and other meat products. Any strain of T. gondii has the potential to cause harm to the fetus (if the mother becomes infected for the first time during pregnancy). Likewise, although an individual may become infected while immunocompetent, recrudescence of the parasite can occur if the individual becomes immunocompromised, e.g., later in life during treatment for chemotherapy. Therefore, pending more information on the real prevalence and infectivity of Toxoplasma in meat products from the industrial swine population of the examined area, public health authorities should implement effective sanitary controls for pest management on the pig farms and should carry out periodic slaughterhouse monitoring of pork safety, according to the European Food Safety Authority recommendations (15).

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