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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE DETECTION OF *TRICHINELLA SPIRALIS* IN MUSCLE TISSUE OF SWINE AND DERIVATIVES

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ABSTRACT

Trichinellosis is an emergent zoonosis in several regions of the world and it is considered a public health problem. Trichinellosis represent one of the most important zoonotic diseases in Argentina. The purpose of this study was to develop a real-time PCR assay with internal control to detect *Trichinella spiralis* DNA in samples of swine muscles and its derivatives. PCR amplification of DNA from muscle samples was performed by real-time PCR with an internal porcine DNA amplification control. The developed PCR assay specifically detects *T. spiralis* showing no amplification with other *Trichinella* genotypes and showed an estimated sensitivity of 0.024 larvae per gram in swine muscle samples. From the 21 samples analyzed, five samples negative by enzymatic digestion were positive by real-time PCR, which demonstrates that this technique is capable of detecting *T. spiralis* in cases when enzymatic digestion cannot. In this work, a new molecular assay with internal control for *T. spiralis* detection was successfully developed.

PRACTICAL APPLICATIONS

Trichinellosis is considered a public health problem in Argentina and also represents an economic problem in porcine animal production and food safety. Due to the predominantly zoonotic importance of infection, the main efforts have focused on the control of *Trichinella* or the elimination of *Trichinella* from the food chain. In our country, most human infections are caused by *Trichinella spiralis*, which is transmitted mainly by the ingestion of raw or undercooked infected swine meat or its derivative products. New controls in order to improve food safety for consumers should be developed. This new real-time PCR assay with internal control shows a substantial increase in diagnostic sensitivity in comparison with muscle artificial digestion and the possibility to avoid false negative results. This specific PCR for *T. spiralis* may be useful for detection of infection at early stages in humans and food animals.

INTRODUCTION

Trichinellosis is an emergent zoonosis in several regions of the world and it is considered a public health problem (Gottstein et al. 2009). It is caused by nematodes of the genus *Trichinella*. Most human infections are caused by *Trichinella spiralis*, which is transmitted mainly by the ingestion of raw or undercooked infected swine meat or its derivative products. In Argentina, the trichinellosis is an important zoonotic disease with 5,217 notified human cases from 1990 to 1999 (Bolpe and Boffi 2001) and 5,820 cases in

2000–2010 (Boletín de Vigilancia 2000–2010). In Argentina, it has been reported *T. spiralis* in domestic, synanthropic and wild animals and *Trichinella patagoniensis* in *Puma concolor* (Krivokapich et al. 2006, 2008, Ribicich et al. 2010, Krivokapich et al. 2012).

The gold standard method recommended by the World Health Organization for diagnosis of trichinellosis is the muscle artificial digestion. Muscle digestion methods involve the enzymatic digestion of muscle tissue and microscopic examination of remaining sediment for free *Trichinella* larvae. Samples can be processed in pools to increase cost-efficiency showing a test sensitivity of ≥ 3 larvae per gram (LPG) when 1 g of domestic pig muscle is analyzed (Forbes *et al.* 1988). In Argentina, a pool of 100 g of tissue is required in the slaughterhouses and the detection efficiency is 1 LPG (SENASA 2010) but it has several sources of error on the level of sedimentation and visualization (Riehn et al. 2011). In Argentininan diagnostic laboratories, 10 g of sample is required for animals suspected of having *T. spiralis* infection.

The enzyme-linked immunosorbent assay (ELISA) is the most commonly used serological test for *Trichinella* detection which is used to monitor the infection by measuring the level of immunoglobulin G proteins (or *Trichinella* antibodies) in serum or muscle juice samples (Uparanukraw and Morakote 1997). Although it is an indirect method and only provides evidence of exposure to *Trichinella* parasites, ELISA assays demonstrate improvement in test sensitivity compared to muscle digestion methods (0.02 LPG in domestic pigs; Gamble 1988); however, it has the disadvantage of showing low specificity due the antigenic cross-reactions with other parasitic infections of the host (Nöckler et al. 2009).

Real-time PCR is a selective amplification of a target region within a DNA to be quantified with fluorescent markers throughout the reaction. It detects the pathogen responsible for infection with high specificity and sensitivity, rapidly (2 h), in large scale, and allows qualitative detection and/or quantitative measurement of parasite DNA (Jauregui et al. 2001; Learmount et al. 2009). Several real-time PCR assays targeting different genes for specific detection of *T. spiralis* DNA have been described. Previous developed specific assays for *T. spiralis* detection were applied to the study of wild life samples (Atterby et al. 2009; Cuttel *et al.* 2012) or to the study of experimental models (Golab et al. 2009; Li *et al.* 2010). Guenter *et al.* developed a real-time PCR methodology for *T. spiralis, Trichinella britovi* and *Trichinella pseudospiralis* DNA detection in domestic pigs infected which showed a sensitivity of 0.1 LPG (Guenther et al. 2008); but there is not background about specific *T. spiralis* real-time PCR performed on muscle samples derived to the diagnostic laboratory.

The objective of this work was to develop a real-time PCR assay with specific newly designed primers and including an internal control for the detection of *T. spiralis* DNA in swine samples and its subproducts which are the most common consumed in Argentina.

MATERIAL AND METHODS

Muscle Samples

A total of 21 muscle samples (16 pig, 3 Wild boars, 1 salted bacon and 1 sausage of wild boar and deer) were first analyzed by enzymatic digestion and then stored at −20C for later analysis by molecular biology. Samples were taken from the predilection sites in pig: fleshy pillars diaphragm, intercostal and masseter muscles and tongue. In the case of wild boars samples consisted in foreleg muscle.

Trichinella Species DNA

DNA purified from all species of *Trichinella* [T1, *T. spiralis* (ISS336), T2, *Trichinella nativa* (ISS140), T3, *T. britovi* (324), T4, *T. pseudospiralis* (ISS176), T5, *Trichinella murrelli* (ISS35), T6, *Trichinella* T6 (ISS34), T7, *Trichinella nelsoni* (ISS29), T8, *Trichinella* T8 (ISS272) T9, *Trichinella* T9 (ISS409) T10, *Trichinella papuae* (ISS1980) T11, *Trichinella zimbabwensis* (ISS1910) T12, provided by the International *Trichinella* Reference Centre (Rome, Italy) and *T. patagoniensis* (ISS2311) provided by INEI, ANLIS Dr. Carlos G. Malbrán (Buenos Aires, Argentina] were used in this study.

Artificial Digestion

The presence of infection and worm burden (LPG) were evaluated by the artificial digestion method from 10 g of each sample (0.7% HCl, 0.5% pepsin, 32C for 3 h) according to standard procedures (SENASA 2010).

MOLECULAR STUDIES

Primer Design

Specific primers to detect DNA of *T. spiralis* were designed using the software Primer Premier 5 (PREMIER Biosoft International, Palo Alto) based on *T. spiralis* mitochondrion complete genome, NCBI Reference Sequence: NC_002681.1. The designed primers (called triqui 133 fw and rv) amplify a 133 bp fragment from the gene ATP6 "ATP synthase subunit 6" of *T. spiralis* DNA. Primers were synthesized at Operon (Huntsville, Alabama).

DNA Extraction of Muscle Samples

Ten grams of each sample were ground using a hand grinder with 90 mL of PBS and DNA was extracted from 1 mL of this homogenate using the commercial kit AxyPrep Multisource Genomic DNA Purification (Axygen, Tewksbury,

MA) and DNA was quantified by Quant-iT PicoGreen dsDNA Assay (Invitrogen, Carlsbad, CA). All the samples analyzed showed a DNA concentration between 10 and 300 ng/μL of DNA, some were diluted for PCR amplifications at concentrations of 10–50 ng/μL, in order to avoid PCR inhibition due to excess of host DNA, as has been previously described (Cuttel *et al.* 2012).

Real-Time PCR Assays

Purified DNA of *T. spiralis* was used as positive control for the validation of the specific real-time PCR for *T. spiralis* detection. Different PCR amplification conditions were tested, by changing variables such as temperature of annealing, primer concentration, number of cycles, DNA concentration and final reaction volume, in order to avoid producing false positives or false negatives. During the validation process, PCR products were analyzed on agarose gels to check the size of the generated amplicons.

The amplification of a fragment of 133 bp of DNA from *T. spiralis* was carried out with the designed primers triqui fw (5′-acattctgaaagacacgat-3′) and triqui rv (5′-gtagggagattaggttgc-3′) in a Rotor Gene Q thermal cycler (Qiagen, Hilden, Germany) in a final volume of 20 μL using EvaGreen as intercalating fluorescent dye (KAPA HRM FAST, Biosystems, Woburn). Rapid cycling program for detection consisted of an initial denaturation of 2 min at 95C, and 45 cycles of 94C 10″, 50C 10″, 72C 15″. After amplification a melting curve of the amplified product was performed being the melting temperature of the specific product of 73.5 ± 1C. In all cases, experiments were done in duplicates. Negative controls were included in all cases. Samples with *Ct* (Cycle Threshold) values below 40 were considered positive.

Internal Control PCR

In order to check the success of DNA extraction and the lack of inhibition, real-time PCR amplification with primers (CERH 5′GCAATACATTACACATCAGACACAA 3′, CER L5′GATGAATAGGCAAATAAAGAATATG 3′) that amplify a 135 pb product of *Sus scrofa* cytochrome b DNA was performed in all the samples (Santaclara et al. 2007). Amplifications were carried out in a Rotor Gene Q thermocycler in a final volume of 20 µL using EvaGreen as intercalating fluorescent dye (KAPA HRM FAST). The cycling program consisted of an initial denaturation of 3 min at 95C, and 35 cycles of 94C 20″, 51C 30″, 72C 30″. After the amplification a melting curve was performed, being the melting temperature of the amplified product of 80.5 ± 1C. Samples with cytochrome b DNA *Ct* values below 35 were considered suitable for further analysis.

Comparison of the Results Obtained by Enzymatic Digestion and Real-Time PCR

To measure the agreement between enzymatic digestion and real-time PCR, the Kappa index was evaluated using Epidat 3.1. A value of kappa higher than 0.75 will indicate excellent agreement while a value lowers than 0.4 will indicate poor agreement.

RESULTS

Real-Time PCR Assays Development

Assay Specificity. A specificity study using as template DNA purified from all genotypes of *Trichinella* (T1-T12) in real-time PCR reactions, showed that only *T. spiralis* amplified the specific 133 bp fragment.

Assay Sensitivity for the Detection of *T. spiralis* in Samples

Once the specificity of the test was confirmed, the sensitivity of the developed technique was studied with purified DNA of *T. spiralis* and also with a sample of pig muscle positive by enzymatic digestion. Analyzing serial dilutions of DNA obtained from a positive sample by enzymatic digestion, it was estimated that the technique developed in this study shows a sensitivity of detection of 0.024 LPG of *T. spiralis* (Fig. 1A,B). The efficiency of the PCR reaction was also determined (Fig. 1B).

Study of the Presence of DNA from *T. spiralis* in Samples

In muscle samples previously analyzed by enzymatic digestion technique, the presence of *T. spiralis* DNA was studied by PCR amplification with primers Triqui 133. A total of 21 different samples (pig, wild boar, sausages, salted) were analyzed. One sample was not suitable for further analysis showing an internal control porcine DNA amplification *Ct* value of 39, which was a sample showing a clear state of putrefaction. This sample was excluded from the final analysis.

Comparison of the Results Obtained by Enzymatic Digestion and Real-Time PCR

Of the 20 samples studied, two samples (Table 1) were positive by enzymatic digestion and were also positive by the real-time PCR assay designed in this study (Table 1). The most remarkable finding was that five samples (two wild boars, two pigs and a sausage of wild boar and deer) negative by enzymatic digestion technique were positive by real-time PCR, demonstrating that the developed technique in the current work is more sensitive than the artificial digestion technique (Table 1). Kappa index was 0.34 ($P = 0.04$)

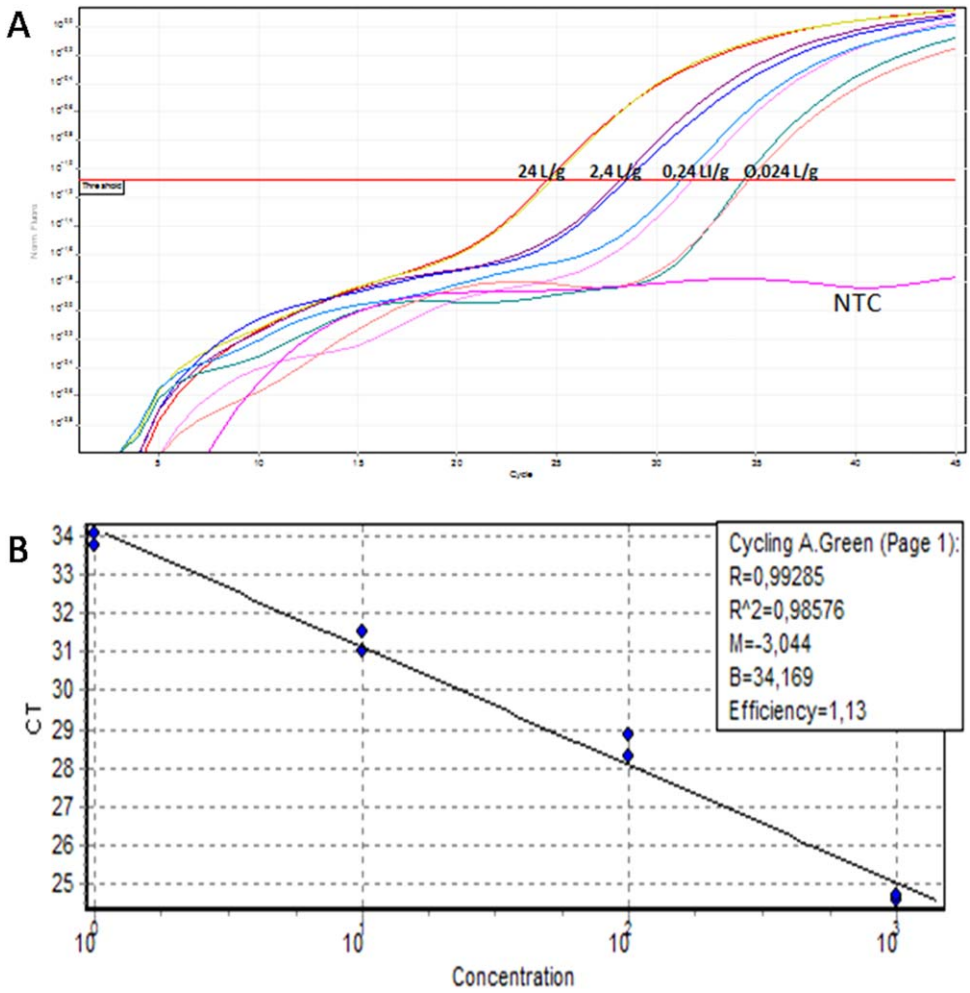

FIG. 1. ASSAY SENSITIVITY. PROTOCOL USING AS TEMPLATE SERIAL DILUTIONS OF A PIG MUSCLE SAMPLE POSITIVE BY ENZYMATIC DIGESTION (24 LARVAE/GR, 2.4 larvae/gr, 0.24 larvae/g, 0.024 larvae/gr)

(A) Dynamic Log range curve for calculating real-time PCR assay sensitivity, presented in a graphic format of increase in fluorescence (delta Rn) on a logarithmic scale plotted against number of cycles. NTC (no template control). (B) Graphical representation of *Ct* values (cycle number) versus the initial concentration of template DNA. On the right box the value of the reaction efficiency is shown.

indicating poor agreement, demonstrating that the differences between techniques are statistically significant.

DISCUSSION

Diagnostic gold standard method for *Trichinella* involves the enzymatic digestion of muscle tissue and microscopic examination of remaining sediment for free *Trichinella* larvae. Samples can be processed in pools to increase cost efficiency, but relevant sources of error in quantitative analyses occur at the following steps of the official procedure: sampling (improper samples and mix-up), sample preparation (inadequate grinding), digestion (unsuitable/expired chemicals, inadequate time/temperature parameters), sifting (sieve size), sedimentation (sedimentation time too short/long) and visualization (erroneous diagnosis) (Riehn et al. 2011). The pooled sample digestion method is generally accepted to have a test sensitivity of ≥ 3 LPG when 1 g of domestic pig muscle is used while a sample size of 5 g enables detection of 1 LPG (Forbes *et al.* 1998).

TABLE 1. SAMPLE ANALYSIS

	Enzymatic digestion (+)	Enzymatic digestion (−)	Total
Real-time PCR +	2	5	7
Real-time PCR −	0	13	13
Total	2	18	20

Comparison of the results obtained by enzymatic digestion and real-time PCR.

Ten grams are needed for individual pig In Argentina, but not always the remitted samples come from the required zone of the animal (diaphragm, tongue and intercostals and masseter muscles) and do not always come in the required amount. Enzymatic digestion is a low sensitivity technique and as it uses only a small portion of the samples, it is likely to have false negatives.

That may be the case of the current work since three samples (two of pig and one of wild boar and deer sausage) were negative probably due to insufficient material, but the high sensitivity of the real-time PCR was able to detect *T. spiralis* DNA.

Molecular detection methods such as PCR are alternative assays that offer both high sensitivity and specificity (Cuttell et al. 2012). In this work, specific primers for detection of *T. spiralis* DNA by real-time PCR were designed. A specificity test against other species of *Trichinella* (T1–T12) was performed and showed that there is no nonspecific amplification with any of the other species of *Trichinella* sp.

All previously described real-time PCR techniques for *T. spiralis* DNA detection lacked an internal amplification control to detect PCR inhibition (Guenther et al. 2008; Atterby et al. 2009; Cuttell et al. 2012). Inclusion of internal amplification controls is mandatory when describing diagnostic tests based on PCR (Hoofar et al. 2003). Since muscle is a known source of PCR inhibitors, it is important that PCR based tests to detect pathogens in this tissue include an internal control. In a PCR without an internal control, a negative response (no amplification) can mean that there was no target sequence present in the reaction. But it could also mean the presence of inhibitory substances in the sample matrix, among other possible reasons (Burd 2010). It is also important, that the fragment length of the internal control PCR is of similar size in comparison with the amplicon generated from the PCR to detect the pathogen, in case of analyzing samples that could be degraded, in this work *S. scrofa* PCR product was 135 pb and *T. spiralis* product 133 pb. This is the first report of a *Trichinella* DNA real-time PCR detection assay including an internal porcine DNA amplification control.

Wild boars and other wild animals remain the main source of trichinellosis throughout the world. In this work, from the three wild boars samples analyzed, two were positive by real-time PCR, also one sample of wild boar and deer sausage was positive. Wild animals may play an important role as reservoirs of *Trichinella* worms in nature and serve as other sources of human infection.

T. spiralis is highly pathogenic and is responsible for most human *Trichinella* infections (Pozio and Murrell 2006). We have developed a real-time PCR technique for the specific and sensitive detection of DNA from *T. spiralis* in muscle from pork, wild boar and its derivatives (sausages and salted) main sources of infection in Argentina. It was estimated that the sensitivity of this developed technique was 0.024 LPG of *T. spiralis* and five samples negative by enzymatic digestion were positive by PCR, which demonstrates that this technique shows a substantial increase in diagnostic sensitivity and the possibility to avoid false negative results since a 35% of the samples had trichinosis, while only 10% have been detected by enzymatic digestion.

It has been suggested that specific PCR for *T. spiralis* may be useful for detection of infection at early stages in humans and food animals that test negative for anti-*Trichinella* antibodies (Li *et al.* 2007). Imperative objective is to continue the development of this diagnostic methodology from samples of blood or serum of live pigs to early epidemiological control and prevention of herds.

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