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Short Communication

# Detection of *Trichinella britovi* in pork sausage suspected to be implicated in a human outbreak in Mendoza, Argentina

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ABSTRACT

Of the three *Trichinella* species described in South America, *T. spiralis*, *T. pseudospiralis* and *T. patagoniensis*, only the former has been implicated in human infections from consumption of pork-derived products. During a presumed trichinellosis outbreak in 2012 in Mendoza, Argentina, we evaluated the serological responses of three patients who had eaten the incriminated food and had signs and symptoms compatible with trichinellosis, using ELISA. We also analyzed potentially contaminated pork sausage by artificial digestion technique and recovered *Trichinella* muscle larvae, which were identified to the species level using a PCR multiplex assay and se-quencing a region of the mitochondrial gene coding cytochrome oxidase subunit I. No antibodies were detected in the sera of the patients, probably because the samples were collected during the immunological window period. According to molecular identification, all larvae from the sausage corresponded to *T. britovi*. *Trichinella britovi* is reported here for the first time in the American Continent, and represents the only cold-tolerant member of the genus in the Neotropics. This species was most likely introduced from Europe to South America during Spanish colonization through pigs, wild boars and/or rats.

Nine species and three genotypes have been described for the genus *Trichinella*. In Argentina, *T. spiralis* is the etiological agent of *Trichinella* infection in humans and pigs [1,2,3] and in the last decade two additional species have been reported, *T. patagoniensis* parasitizing *Puma concolor* [4], and *T. pseudospiralis* found in a domestic pig from the Patagonian region [5]. Up to now, only *T. spiralis* has been implicated in human infections from the consumption of pork-derived food products. In the present work we describe the analysis of serum samples of patients with clinical symptoms and food suspected as the source of infection in an outbreak of trichinellosis that occurred in Argentina.

In July 2012, about 15 patients apparently became infected through the consumption of sausage, bacon and/or ham contaminated with *Trichinella* in Las Heras City, Mendoza, Argentina (68°50′W, 32°51′S). Unfortunately, clinical histories and blood samples for laboratory tests were available from only three patients, one man aged 20 years and two women aged 32 and 36 years. Clinical examination indicated signs and symptoms compatible with trichinellosis such as fever, myalgia, facial edema and conjunctivitis; additionally, the male patient presented

exanthema and was hospitalized for 4 days. The three patients claimed to have consumed the suspicious food on July 20. Blood samples were taken on July 27 from the man and on August 6 from the women. Clinical manifestations were recorded by the physician on the date of blood withdrawal for the man and the 36-year woman and on July 29 for the 32-year woman. All three of them showed eosinophilia $(3.69–5.07 \times 10^6$ cells/ml) and high levels of CPK (range 394–446 U/L, normal reference value $\leq 195$ U/L) and LDH (range 558–757 U/L, normal reference value $\leq 450$ U/L). They were treated with albenda-zole (400 mg twice daily for 10 days). Serodiagnosis was conducted by an enzyme-linked immunosorbent assay (ELISA) on polyvinyl micro-plates (MaxisorpTM) sensitized with 50 μg of excretory-secretory anti-gens from muscle larvae of *T. spiralis* (ISS643). Plates were incubated with serum samples (diluted 1:250) for 30 min at 37 °C with 0.05% PBS/Tween 20 containing 3% albumin, and then washed three times with PBS/Tween 20 at 0.05%. Specific antibodies were detected using horseradish peroxidase-conjugated goat anti-Human IgG (Invitrogen, USA). Color reaction was developed by adding a solution containing

ortho-phenylenediamine (OPD) and $H_2O_2$ in citrate buffer at pH 5.0. Absorbance values were read on an ELISA automated microplate reader (BIO-TEK Instruments, USA) at 450 nm.

For the validation of the in-house ELISA, the optimum concentrations/dilutions of the antigen adsorbed to plate, serum, enzyme-antibody conjugate and substrate solution were determined through the use of chessboard/checkerboard titrations [6]. The cut-off value was calculated by Two-graph receiver operating characteristics (TG-ROC) curves using negative sera (from asymptomatic blood donors living in non-endemic areas) and positive sera (from clinically and serologically diagnosed patients with a compatible epidemiological history and who consumed food detected positive by artificial digestion). The cut-off value of Elisa gave a sensitivity of 100% and a specificity of 90.4%.

The early detection of anti-*Trichinella* IgG is hindered by the existence of a "blind window", during which seroconversion is delayed for some weeks after infection. Notwithstanding this, diagnosis was based on a single blood sample because patients refused to return for follow-up.

A sample of a pork sausage suspected of being the source of the infection was examined for muscle larvae by the artificial digestion magnetic stirrer method [7].

Genomic DNA was purified from a pool of *Trichinella* L1 larvae using QIAamp DNA Blood Mini Kit (Qiagen). Identification to the species level was performed by a multiplex PCR of nuclear ribosomal DNA [8] and by nucleotide sequencing of the mitochondrial cytochrome C oxidase subunit I gene (COI). A COI fragment of 363 bp was amplified by PCR using the primers 5´AGAAGTATACATTCTGGTGTTACCTGCTT -3´ and 5´- GAGGCATTTGAGAGTCTAACTCCTG-3´. The DNA sequence was directly sequenced using the same primers as for amplification and deposited (without primer regions) into the GenBank database under the accession no. MG570049. Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) against non-redundant nucleotide database at NCBI (www.ncbi.nlm.nih.gov/blast/).

Sera from all three patients were negative for *Trichinella* by ELISA test.

The food sample was positive for *Trichinella,* with a burden of 4 larvae/g. All the examined muscle larvae were not motile and displayed a C-shaped morphology, characteristic of non-viable specimens. Their loss of viability was possibly due to the pronounced desiccation of the analyzed pork sausage.

Molecular identification by multiplex PCR showed a pattern of two bands of 127 bp and 253 bp corresponding to *T. britovi*. Likewise, BLAST search revealed that the COI DNA sequence from the isolate showed strong homology (100–99%) with COI sequences of *T. britovi* (GenBank Accession nos. KM357413, MF402920, AF129488, DQ007892, KY464997). Therefore, analysis using markers of nuclear ribosomal and mitochondrial DNA sequences identified *T. britovi* as the species implicated in the trichinellosis outbreak that occurred in Las Heras, Mendoza, in 2012.

The patients' clinical findings, their eosinophilia and high levels of CPK and LDH enzymes strongly suggest that they were undergoing the acute phase of trichinellosis. Blood tests suggesting acute trichinellosis were performed 7 and 17 days after the man and the women, respectively, consumed the suspected food, while the clinical manifestations for the man, the 32-year woman and the 36-year woman were recorded 7, 9 and 17 days after its consumption, respectively. It is worthy to note that for the man, the results of the laboratory tests and clinical manifestations were compatible with a *Trichinella* infection already at day 7 post-ingestion. The newborn larvae of *Trichinella* are released into the circulatory system from day 5 post-infection [9]. These migrating larvae and their metabolites trigger an immediate reaction involving not only pathological and metabolic disorders, but also an immunological response characterized by higher levels of infiltrating inflammatory cells [10]. Such an early response has been previously reported in human trichinellosis outbreaks, with eosinophilia usually appearing early and before the onset of clinical signs and symptoms, and increasing between the second and fifth weeks of infection [11]. This may explain the early eosinophilia in the man. On the other hand, muscle enzymes are generally detected from the second week onward [11]. The earlier-than-usual detection of muscle enzymes in the male patient may have been due either to a particular individual response to the infection or to the fact that the suspected food was eaten before the date reported by the patient. The three studied cases showed clinical signs and symptoms that have been seen from the first week post-infection onward, such as fever, myalgia and facial edema [10]. In particular, the male patient manifested symptomatology as early as 7 d post-infection, in agreement with that observed in other trichinellosis outbreaks. The patients also had other clinical signs commonly associated with acute trichinellosis, such as conjunctivitis in all three cases and exanthem in the man. Moreover, none of them reported gastrointestinal signs, which have sometimes been observed about 2 d post-infection [10,11].

The seronegativity of the single blood sample from each patient likely indicates that the latter were in the immunological window period, considering that blood collection was accomplished 7 and 17 days after the consumption of the suspected food and that *Trichinella* infection may remain immunologically silent for some weeks [10]. Overall, all these data suggest the occurrence of an outbreak caused by *T. britovi* en Argentina.

It is the first report on the molecular identification of *T. britovi* in Argentina. Until now, *T. britovi* was known to occur in Europe, northwestern Africa and southwestern Asia [12]; thus, this species is reported herein for the first time in the American Continent. In addition, it represents the fourth species of genus *Trichinella* described from Argentina, along with *T. spiralis*, *T. pseudospiralis* and *T. patagoniensis* [1–5].

Larvae of *T. britovi*, the Holarctic species *T. nativa* and the Nearctic genotype *Trichinella* T6 are cold-resistant and can survive longer in frozen muscles of carnivores [10]. Therefore, the detection of *T. britovi* in the Neotropics suggests that, contrary to what was believed until now, *Trichinella* transmission may also occur under low-temperature conditions in Argentina. Therefore, our finding indicates that the freezing method should not be used for the control of *Trichinella* in wild animals intended for human consumption in this region, reinforcing the recommendations of the International Commission on Trichinellosis (http://www.trichinellosis.org/uploads/Post-Harvest_Control_3-13-17.pdf).

The source of infection was attributed to hand-made products elaborated from a domestic pig most likely born and raised in Argentina and slaughtered in the City of Las Heras without veterinary control. Indeed, there was no evidence that this food came from overseas. *Trichinella britovi* may have been introduced from Europe to South America during Spanish colonization through pigs, wild boars and/or rats, as it was speculated for *T. spiralis* and *T. pseudospiralis* [5,13]. In the Old World, swine infection is 1.9 times more frequent by *T. spiralis* than by *T. britovi* [12]. In Argentina, the molecular identification of over 200 *Trichinella* isolates from domestic pigs and their products from different provinces indicated that they belonged to *T. spiralis* except for one, which was assigned to *T. pseudospiralis* ([1,3,5] https://trichinella.iss.it/Trichinella/Search]).

Despite the large numbers of isolates analyzed so far in Argentina, the single detection of *T. britovi* may be due to climatic conditions, which limit its transmission but not that of the cosmopolitan *T. spiralis*. Further research is required to evaluate the actual extent of circulation of *T. britovi* among animal hosts in the studied region.

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## Declaration of interest

None.

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