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Trichinella infection in wild boars (*Sus scrofa*) from a protected area of Argentina and its relationship with the presence of humans

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ABSTRACT

In Argentina, *Trichinella* infection has been documented in humans and animals of several provinces since 1930. This zoonotic parasite infection has been recently detected in humans and pigs of a region historically considered as *Trichinella*-free, suggesting the spread of these pathogens. The aim of the present work was to investigate the presence of *Trichinella* infection in wild boars (*Sus scrofa*) and in the human population living in a protected area. *Trichinella* infection has been investigated by serology (in humans and wild boars) and by artificial digestion of wild boar muscles. The isolated *Trichinella* larvae have been identified at the species level by multiplex PCR. A geographical information system has been used to collect environmental data. The results showed the circulation of *Trichinella spiralis* in wild boars with a low parasite burden, and suggest the influence of human behavior on the transmission. The transplacental passage of parasite is postulated. It follows that the declaration of region as *Trichinella*-free should be carefully established by means of extensive monitoring programs, not only in humans and domestic animals but also in wildlife.

1. Introduction

Trichinellosis is a worldwide zoonotic disease caused by nematode worms of the genus *Trichinella* (Dupouy-Camet and Murrell, 2007). These parasites circulate among domestic pigs and wild animals (both carnivores and omnivores) and can reach the human beings through the consumption of raw or undercooked meat bearing larvae of *Trichinella* spp.

In Argentina, *Trichinella spiralis* is the most prevalent etiological agent found in domestic pigs and synanthropic animals (e.g. brown rats and armadillos); however, little is known about its circulation among wildlife, even when outbreaks of trichinellosis caused by the consumption of meat from wild animals have been documented (Tesón et al., 1997; Huici et al., 1999; Pozio, 2000; Ribicich et al., 2005).

Today in Argentina, there are endemic, non-endemic and *Trichinella*-free provinces (Bolpe and Boffi, 2001). The criterion adopted by the local health authorities for this classification is based on the presence or absence of human or porcine outbreaks or the detection of isolated infections.

The presence of a transmission cycle of the parasite among domestic animals in a given region is frequently associated with the presence of infection in wildlife (Pozio et al., 1996; Pozio, 1998). In Europe and in non-European countries, the wild boar plays an important role as reservoir for *Trichinella* spp., mainly *T. spiralis* and it is

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also one of the most important sources of infections for humans (Pozio, 2007; Pozio et al., 2009).

Recently, some of us have shown the presence of *Trichinella* infections in humans and pigs of an area which had been previously considered to be free from these zoonotic parasites (Costantino et al., 2009). Taking into account the high percentage of humans and swine that were serologically positive in this area, we looked forward to detect the presence of these parasites in wild boars. The aims of the present work were (1) to detect *Trichinella* infection in wild boars and humans by means of parasitological and/or serological methods, (2) to identify the parasite species circulating in the area, and (3) to establish the geographical distribution of the wild boar infection and its relationship with the presence of human settlements.

2. Materials and methods

2.1. Area under study

The area under study belongs to a natural protected habitat belonging to National Park Administration, located in the center-east of the Entre Ríos province of Argentina, along the occidental riverside of the Uruguay River (31°51′44″S; 58°13′57.4″W). The park has an extension of 8500 ha featured by a slightly undulating plain landscape. The climate is variable and the vegetation consists mainly of low woods and savannahs alternating with hard grasslands and palm trees. The fauna consists of native species such as rodents (plains viscacha, *Lagostomus maximus*; capybara, *Hydrochoerus hydrochaeris*), birds (rheas, *Rhea americana* and *R. pennata*) and several species of lizards which co-inhabit with exotic species such as the axis deer (*Cervus axis*), several antelope species and wild boars.

The park is divided by a creek into two areas: the Northern area, where a recreational zone (with camping, restrooms, a drugstore, barbecues, a restaurant, the governor's office and a beach) is located, and the Southern area where hunting activities are permitted only for authorized persons.

The nearest human settlement is 12 km far from the entrance of the park. This village has 2334 inhabitants (INDEC, 2001) most of them devoted to agricultural activities, and being some others employees at the park. Some of these inhabitants are authorized hunters and the game meat originating from the hunting activity is served in communal canteens of the village. Hunters coming from other villages or towns are allowed to take away part of the game meat for themselves. An important feature of the village is the presence of a huge open air garbage dump located 6 km far from the park, separated by the N°14 National Route and to which it is fairly easy to gain access.

2.2. Samples

Muscle samples originated from 112 adult wild boars, a 3-day old wild boar and from a fetus all of them hunted at random in 2007. Serum samples were collected from 48 wild boars by puncture of the femoral vein. In addition, serum samples were also collected from 44 people (31 men and 13 women). Written consent was obtained from all the blood donors. These people (hunters, rangers and park personnel) belong to a human settlement located in the park. All serum samples were kept at −20 °C until used.

2.3. Collection of epidemiological data

To evaluate the alimentary habits at risk for this parasite infection, a questionnaire was given to enrolled people to get information about the frequency of game consumption and how the meat was consumed (raw, semi-raw, etc.). In addition, age, gender, occupation, and the possible occurrence of signs and symptoms of trichinellosis after game consumption, were included in the questionnaire.

2.4. Parasitological test

Samples of skeletal muscle (diaphragm, tongue, masseters and hind quarters) weighing 85 ± 22 g were tested from each wild boar ($n = 114$) by the artificial digestion (AD) according to a previous published protocol (Gamble et al., 2000). After digestion and sedimentation, *Trichinella* larvae were collected, washed in saline and counted by a light microscope and by two independent observers. Results were expressed as number of muscle larvae per gram of digested muscles (ML/g). Larvae were preserved in absolute ethanol and sent to the International *Trichinella* Reference Centre at the Istituto Superiore di Sanitá (Rome, Italy) to be identified at the species level by Multiplex PCR according to a published protocol (Pozio and La Rosa, 2003).

2.5. Detection of anti-Trichinella antibodies in sera

The detection of anti-*Trichinella* antibodies in human serum samples was carried out by ELISA, indirect immunofluorescence (IIF) and Western blot (WB), as previously described (Nuñez et al., 2000; Calcagno et al., 2005). Similar tests, developed for the detection of anti-*Trichinella* antibodies in porcine serum samples, were used to detect anti-*Trichinella* antibodies in sera from wild boars, with slight modifications (Venturiello et al., 1998; Nuñez et al., 2000). Briefly, all anti-swine IgG (H + L) sera were previously tested to corroborate their reactivity with wild boar sera. For the IIF, serum samples were employed at a dilution of 1/10 in saline with 0.1% Tween and an anti-swine γ-globulin serum conjugated to fluorescein isothiocyanate (FITC, Dako Corporation, Carpintería, CA, USA) diluted 1/70 in 1/30000 Evans blue. Those samples rendering fluorescence of the cuticle of the parasite were considered positive. In the WB, an anti-swine γ-globulin serum conjugated to biotin (Vector Laboratories, Burlingame, CA, USA), diluted 1/70 was employed followed by the addition of a macromolecular complex of avidin and biotinylated peroxidase (ABC, Vector Laboratories) according to the manufacturer's instructions. Those samples developing the characteristic bands corresponding to the molecular weights of 45 and 55 kDa were considered positive. For ELISA, an anti-swine γ-globulin serum conjugated to biotin (Vector Laboratories) diluted 1/

1000 was used followed by the addition of the macromolecular complex of avidin and biotinylated peroxidase (ABC, Vector Laboratories). Animals with a negative result by AD, IIF and WB were used as negative controls. Samples with an OD value greater than 2.5 times the average OD values of the negative controls were considered to be positive.

2.6. Geographical data

The locality of origin of hunted wild boars was determined by a GPS device. Longitude and latitude with other data were analyzed in a geographical information system (GIS; Geomedia Professional 4.0) to produce thematic maps of age, sex, weight and presence of the infection. The hypothesis that the *Trichinella* infection in wild boars was associated with the human settlements (with the presence of a garbage dump or with a recreational area), was tested. The area under study was divided into two concentric circles termed "buffer areas" and which delimited an inner zone having a radius of 6 km containing the recreational area and an outer zone having a radius of 12 km which corresponded to the distance between the inner zone and the park entrance. Data were analyzed by contingency tables employing the Fisher's test and the relative risk was evaluated. The statistical analysis was performed with the Graph Pad Prism 4.0 software.

3. Results

Thirteen out of 114 wild boars studied (11.4%) were positive by AD, having 10 of them (76.9%) a parasite burden ranging from 0.01 to 0.03 ML/g and 3 of them (23.1%) a parasite burden ranging from 0.2 to 0.3 ML/g. Two of these positive animals were a 3-day-old wild boar and a fetus which had a parasite burden of 0.2 ML/g and 0.01 ML/g, respectively. Larvae collected from muscle samples of three adult wild boars were identified as *Trichinella spiralis* by molecular analysis. Larvae collected from the muscles of the 3-day-old wild boar and from the wild boar fetus did not show any PCR amplification, suggesting that the DNA was destroyed.

Of the 13 parasitologically positive animals, serum samples were available for only 4 of them which resulted positive for the presence of anti-*Trichinella* antibodies. Of the 48 serum samples from wild boars tested by the three serological methods (Costantino et al., 2001; Venturiello et al., 2009), 23 (47.9%) were positive.

A significant percentage (76%) of the total number of wild boars hunted in 2007 ($n = 150$) at the National Park area was sampled. The four wild boars which had resulted to be both serologically and parasitologically positive originated from the inner zone which contains the recreational area. No relationship was found between *Trichinella* infection and other features such as age, sex or weight (data not shown).

No relationship was observed between *Trichinella* infection in wild boars and the proximity to the village (data not shown). The highest prevalence of parasitologically positive wild boars (16%) was found inside the inner zone, whereas this prevalence drops to 4.4% in the outer zone ($p < 0.01$) (Fig. 1). The relative risk of infection in the inner zone compared with the outer zone was 3.6.

The age of the enrolled people ranged from 20 to 60 years (average 36 years for men and 38 for women). Anti-*Trichinella* antibodies have been detected in serum samples

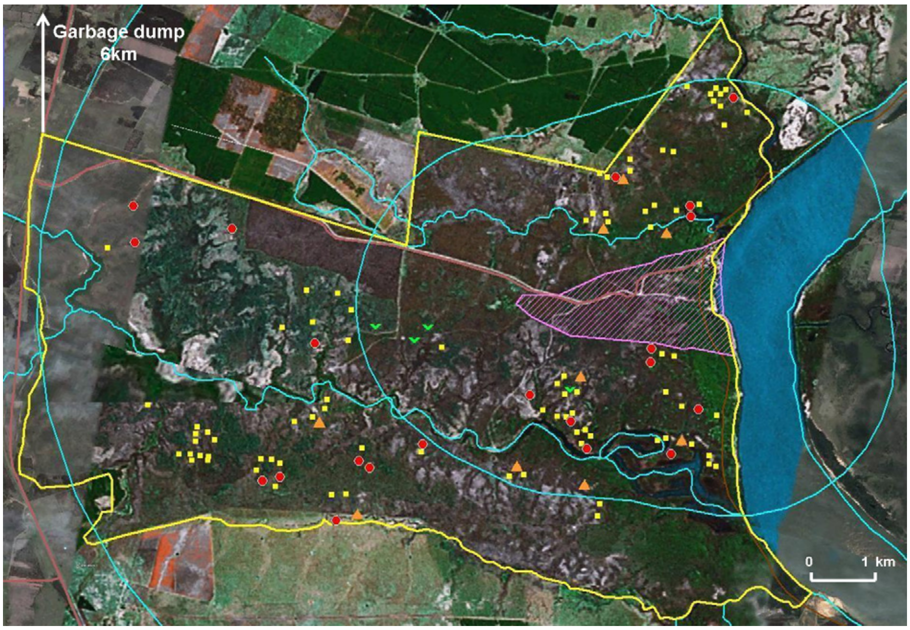

Fig. 1. Map of El Palmar National Park, Entre Ríos, Argentina. The localities of origin of hunted wild boars (*Sus scrofa*) are shown. Serologically positive wild boars (red dots), parasitologically positive wild boars (orange triangles), serologically and parasitologically positive (green arrows) or negative (yellow squares) wild boars. Recreational area (pink stripes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

from two people (4.5%) by more than one serological method. These two samples belonged to female persons who declared to consume undercooked wild game meat. According to the questionnaire, 35 people (79.5%) consume raw wild boar meat or as a raw derivate. Of the 31 people who gave answers regarding the frequency of consumption, 74.2% declared to consume wild boar meat from time to time, 12.9% often and a 12.9% did not consume it whatsoever. None of the individuals referred to have had any signs or symptoms of trichinellosis after the wild boar meat consumption.

4. Discussion

The results show for the first time in Argentina, the presence of *T. spiralis* in an area which has been previously considered to be *Trichinella*-free. These results also suggest the influence of human behavior on the transmission of *T. spiralis* to wild boars.

We have recently demonstrated the existence of human trichinellosis and infection in swine in the province to which the area under study in this work belongs to. It is noteworthy that this province has been historically considered to be free of *Trichinella* (Costantino et al., 2009). The latter results, together with those presented here, are in line with previous epidemiological works (Pozio, 1998; Peréz–Martin et al., 2000) which show how domestic pigs can be the source of *T. spiralis* infection for wild boars and vice versa. All parasitologically positive animals presented low parasite burdens, suggesting a trend, in wild animals, towards low infection intensity as observed in other studies (Peréz–Martin et al., 2000; Malakauskas et al., 2007).

By the parasitological method, a lower percentage of animals could be diagnosed, as compared with the figures obtained by serology. It is noteworthy that these infections would not be detected by routine meat inspection which is usually performed with 10 g samples (Gamble et al., 2000). This finding correlates with the low parasite burden (Venturiello et al., 1998), even if false positive results cannot be excluded due to cross reactions with other parasite antigens as already observed in other serological studies (Gómez-Morales et al., 2008). However, the simultaneous use of two or more serological methods allows being confident that the serologically positive animals harbored some *Trichinella* larvae.

Despite the low parasite burden found in the wild boars, seropositive findings in humans indicated an exposure to *Trichinella* larvae, however, no clinical signs of trichinellosis were observed.

Experimental infections have shown that vertical transmission occurs in ferrets, guinea pigs, and in mice, rats and rabbits (Podhájecký and Tomasovicová, 1968; Cosoroabã and Orjanu, 1998; Nuñez et al., 2002; Webster and Kapel, 2005). No vertical transmission was observed in red foxes and pigs (Webster and Kapel, 2005). In humans, the vertical transmission has been postulated (Dubinský et al., 2001; Nuñez et al., 2008). The presence of nematode larvae, identified as belonging to the genus *Trichinella* by their morphology and size (a worldwide accepted criterion for the detection of infection), in muscles of a 3-day-old wild boar and of a wild boar fetus opens the question if, in particular circumstances, some larvae can be transmitted from the mother to the fetus. Further investigation should be done to confirm or not the occurrence of a vertical transmission in wild boars, due to the low number of offspring analysed and the lack of molecular identification. Even if this question is still open, a definitive answer will be of great biological and epidemiological importance in terms of the transmission and dispersion of the parasite.

According to the GIS studies the serologically and parasitologically positive animals were distributed in the surrounding areas of the recreational zone (human settlement) and not in the open air garbage dump of the nearby town (Fig. 1). This observation suggests an infection flow from domestic to sylvatic swine. This speculation lies on the knowledge that the wild boar is a species which has a high degree of tolerance to the human presence allowing its pasture in environments highly influenced by humans (Murrell and Pozio, 2000; Rosell et al., 2001). It has been shown that an improper handling of domestic and wild swine carcasses by humans who scatter pork scraps in the environment, without to follow the sanitary regulations, allows the spread of *Trichinella* infection among wild boars (Pozio and Murrell, 2006).

The increasing use of game meat by hunters and also by town-dwellers, there is a risk of *Trichinella* transmission to humans. The findings of *Trichinella* infection in domestic pigs, wild boars and humans of an area considered to be *Trichinella*-free suggest the urgent need to implement prophylactic measures and to educate the human population on the correct disposal of pork scraps.

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