Porcine and rodent infection with *Trichinella*, in the Sierra Grande area of Río Negro province, Argentina

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In 2000, two cases of human trichinellosis were detected in the Sierra Grande area of Río Negro province, Argentina. As part of an investigation of the aetiology of these cases, 300 pigs slaughtered for consumption in the area between 2000 and 2002 were checked for *Trichinella* infection, by artificial digestion of a muscle sample. Twelve (5.6%) — four (7.3%) of the 55 checked in 2000, five (4.8%) of the 105 investigated in 2001, and three (2.1%) of the 140 investigated in 2002 — were found infected. Blood samples were collected from other pigs aged >6 months old, so that sera could be tested, in ELISA and by western blotting, for anti-*Trichinella* antibodies. Of the 181 animals checked in the initial serological survey, 36 (19.9%) were found seropositive for *Trichinella*. When 35 of the seronegative pigs were re-checked 6 months later, three (8.6%) were found to have seroconverted.

Four (15.4%) of 26 local rodents, caught in Sherman-type traps, were also found positive when checked for infection by artificial digestion. It appears that about 20% of pigs in the study area are infected each year, this high level of transmission being sustained by a high prevalence of infection in the local rodent populations.

Trichinellosis is a zoonosis that is broadly distributed throughout the world. The causative agents, nematodes of the genus *Trichinella*, are mainly to be found infecting carnivorous or omnivorous mammals, including domestic pigs (Rodríguez Aruzco *et al.*, 1991; Gamble, 2000; Sequeira *et al.*, 2000; Geerts *et al.*, 2002).

In the province of Río Negro in central Argentina, there is long history of both porcine and human trichinellosis (Larrieu, 1981). The Sierra Grande area, in the south–eastern corner of the province, has been particularly badly affected by the disease, which remains locally endemic.

When, in 1984, staff of the veterinary service of the Río Negro health secretariat checked pigs held in breeding pens in this area, so many of the animals were found infected with *Trichinella* that the area was officially declared severely affected by trichinellosis (unpubl. obs.). This declaration prompted the intervention of the National Animal Health Service and the culling of every pig (>600 animals) in the area. Microscopical examinations (i.e. trichinoscopies) revealed that 22% of the pigs killed were infected (unpubl. obs.). Since 1985, the local pig population has slowly grown back to its former size, with new introductions from several regions of Argentina. In the last 20 years, however, the human population has declined (from 12,000 residents in 1985 to <6000 in 2000) as a result of the closure of

the iron-ore mining and processing plant that provided the main source of employment. As the current level of unemployment exceeds 50%, life for the local inhabitants is generally very difficult, with much social deprivation. The pigs in the area are owned by small-scale producers whose primary aim is the production of pork for their families to eat, although some of the meat is sold or exchanged for other goods. Little of the meat is processed into salami or ham, to extend its shelf-life. Fresh pork sausages are marketed in a few butchers' shops.

In 2000, clinicians at the hospital serving the Sierra Grande region reported two cases of human trichinellosis (unpubl. obs.). The National Animal Health Service ordered all of the pigs in the two pig-breeding pens subsequently identified as the geographical sources of these human infections to be culled. When the slaughtered pigs were checked for *Trichinella*, by 'artificial' digestion of muscle samples (Gamble, 1998), 10% were found to be infected (unpubl. obs.).

The main aim of the present study was to evaluate the general prevalence of *Trichinella* infection in the pigs and rodents of the Sierra Grande area, and so gain some insight into the epidemiology of trichinellosis in this and similar, endemic settings.

ANIMALS AND METHODS

Study Area
The study area lies in the Patagonian steppe and is characterized by low rainfall (<200 mm/year), high summer temperatures, and very low winter temperatures. The local water resources, both surface and underground, are very limited.

Pig Census and Control
In October 2000, all the pigs in the Sierra Grande area were located, counted and aged. The older animals (those aged >6 months) were identified with numbered tags, each tag being placed in a left ear. Subsequently, a monitoring system was put in place so that the location of each tagged pig could be updated, as necessary, and any pigs entering or leaving the area could be identified.

A written agreement was made with the pig owners to the effect that any pig found seropositive for *Trichinella* (see below) would be confined to its breeding pen. Breeding pens with seropositive swine were inspected systematically by the veterinary service of the local hospital.

Immunodiagnosis
A blood sample was collected from the central caudal vein of each pig tagged in the census. The sera were separated off, by centrifugation, and then kept frozen at −20°C until they could be checked for anti-*Trichinella* antibodies in ELISA and western blots (Ruitemberg *et al.*, 1975). Six to 23 months later, some of the pigs that had been found seronegative and two of the pigs (confined to their pens) that had been found seropositive were re-tested.

ANTIGEN PREPARATION
The larval excretory–secretory products used as antigens for the serological tests were prepared from *T. spiralis* ISS643 that had been maintained in CF1 mice (Su *et al.*, 1990). Larvae were recovered from infected murine muscle (from mouse carcasses that had been skinned, eviscerated and ground) by digestion with 1% pepsin in 1% HCl for 3 h at 37°C. They were washed in Dulbecco's modified Eagle's medium (DMEM) with penicillin (500 U/ml) and streptomycin (500 µg/ml), and then incubated, for 18 h at 37°C in 5% CO_2, in DMEM supplemented with 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES), 2 mM glutamine, 1 mM pyruvate, penicillin (50 U/ml) and streptomycin (50 µg/ml). The larvae were then removed, by filtering the medium through a 0.2-µm-pore membrane. The filtrate was concentrated under pressure, with a 10-kDa molecular-weight retention. The protein concentration in a

sample of the concentrated filtrate was estimated by the method of Bradford (1976). The remainder of the concentrated filtrate, used as the antigen source for the serological tests, was maintained frozen at $-20°C$ until used.

ELISA

Flat-bottomed, polyvinyl, microtitre plates (MaxiSorp™; Nunc, Roskilde, Denmark) were coated with antigen (5 μg/ml, and 100 μl/well) in carbonate–bicarbonate buffer, pH 9.6, overnight at 4°C. Each well was then washed three times with wash buffer (phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20). The wells were then blocked with blocking buffer (3% albumin in wash buffer) for 30 min at 37°C. After three more rinses with wash buffer, 100 μl of a test serum, diluted 1:250 in blocking buffer, were added to each well and the plates incubated 30 min at 37°C. Then a horseradish-peroxidase conjugate of rabbit anti-pig-IgG (whole molecule; Sigma) in blocking buffer was left in the wells for 30 min at 37°C. After a final three washes, 0.04% ortho-phenylendiamine and 0.04% H_2O_2 in citrate–phosphate buffer (pH 5) were added as substrate. After incubation for 5 min in a dark box, the optical densities (OD) of the well contents were read at 450 nm, in an automated microplate reader. To establish a threshold for seropositivity, 120 sera from pigs found negative for *Trichinella*, by the digestion of muscle samples (see below), were run in the ELISA; the value calculated by adding three S.E. to the mean OD for these sera was used as the threshold.

WESTERN BLOTS

The proteins in a sample of the extract of excretory–secretory antigens were separated by electrophoresis, using a Miniprotean slab cell (Bio-Rad, Hercules, CA), and transblotted onto nitrocellulose membrane at a constant voltage for 1 h. The membrane was left overnight in blocking solution (phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20 and 5% milk), before being cut into 2-mm-wide strips. Each strip was then shaken for 1 h at room temperature with a test serum diluted 1:100 in blocking solution. After three, 10-min rinses with wash buffer, the strips were shaken for 1 h at room temperature with the same peroxidase conjugate as used for the ELISA, then rinsed twice, for 10 min each, with washing buffer, and once, for 10 min, with pure phosphate-buffered saline (PBS). Finally, a substrate solution based on 3,3′-diaminobenzidine (12 mg 3,3′-diaminobenzidine, 20 ml PBS, and 20 μl H_2O_2) was used to reveal the reactive bands. Sera that bound to three antigens, of 49, 52 and 54 kDa, were considered blot-positive for *Trichinella* (Su *et al.*, 1990).

Pig Parasitology and Muscle Digestion

A sample of diaphragm muscle was collected, post-mortem, from each pig slaughtered for consumption between 2000 and 2002. At the local hospital, a 10-g subsample of each sample was digested with pepsin/HCl (as used for the antigen preparation) before being checked under a microscope for *Trichinella* larvae. The samples that appeared larva-positive were sent to the Malbrán parasitology laboratory in Buenos Aires, for confirmation and for typing a few of the parasites (see below).

Similar methods were used to investigate the pigs found ELISA-positive (which were killed but not consumed), although, if no larvae were found in a 10-g subsample, ever-larger samples were used until larvae were observed or a 125-g sample had been checked.

TYPING OF PARASITES

A slight modification of the nested PCR described by Zarlenga *et al.* (1999) was used to identify the *Trichinella* in each sample to species level. DNA was extracted from single larvae, each larva being placed in 5 μl lysis buffer [10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.45% Tween-20, 0.45%

NP-40, 0.01% gelatin, and 0.2 mg protein-ase K/ml], overlaid with a drop of mineral oil, and then heated, first for 90 min at 65°C and then for 15 min at 90°C, before being stored at −20°C until tested.

Each 50-μl reaction mixture for the first-round PCR contained 2.5 μl of DNA extract, 5 μl 10 × PCR buffer, 0.4 μl of a 2.5 mM solution of each deoxynucleotide triphosphate, 0.6 U *Taq* polymerase, and 0.05 μM of each primer (5′-TCT TGG TGG TAG TAGC and 5′-GCG ATT GAG TTG AAC GC). After denaturation for 3 min at 94°C, the DNA was amplified for 25 cycles, each cycle consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, before a final extension for 10 min at 72°C.

For the second-round PCR, each 50-μl reaction mixture contained 2.5 μl of the first-round amplification products, 5 μl 10 × PCR buffer, 0.4 μl of a 2.5 mM solution of each deoxynucleotide triphosphate, 1.25 U *Taq* polymerase, and 0.25 μM pmol of each primer (5′-GTT CCA TGT GAA CAG CAGT and 5′-CGA AAA CAT ACG ACA ACT GC). After denaturation for 3 min at 94°C, the reaction was cycled 40 times, each cycle of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, before a final extension for 10 min at 72°C.

The final reaction products were separated by electrophoresis in 2.5% (w/v) agarose gel and visualized, by trans-illumination with ultra-violet light, after staining with ethidium bromide. Reference isolates of the muscle larvae of *T. spiralis* (ISS599), *T. nativa* (ISS532), *T. britovi* (ISS447), *T. pseudospiralis* (ISS13), *T. murrelli* (ISS103) and *Trichinella* T6 (ISS34) were used as controls.

Rodent Collection and Parasitology

Wild rodents in the study area were caught alive, using Sherman-type traps set in the pig-breeding pens (661 trap-nights) or in the municipal rubbish dump (441 trap-nights). Each rodent caught was killed, skinned and eviscerated. Each carcass was then chopped up and digested with pepsin/HCl to release any *Trichinella* larvae present. One of the larvae released was identified to species using the nested PCR.

Infection in the Human Population

A serum sample was collected from each of the 112 patients who presented at the Sierra Grande hospital over a 7-day period in 2001, for the treatment or diagnosis of conditions other than trichinosis (no symptomatic cases of human trichinosis were detected in the area in 2001 or 2002). These sera were tested for anti-*Trichinella* antibodies, using a similar ELISA to that used to test the porcine sera.

RESULTS

Pig Census

During the 2000 census just 27 pig-breeding pens were located. Together, these pens held 287 pigs (182 piglets aged ≤6 months and 105 older animals that were tagged). Each pen held a mean of 3.2 sows.

Of the 27 breeding pens located, three (11.1%) were in urban areas of the main town, 20 (74.1%) were on the outskirts of this town (100–1000 m from urban areas), and four (14.8%) were in rural areas 5–20 km from the town, on sheep farms or cattle ranches.

Porcine Infection

SEROPREVALENCE

In 2000, sera were collected from 95 (90.5%) of the 105 live pigs aged >6 months, and 27 (28.4%) of these samples were found positive in the ELISA (18) or by western blotting only (nine). Four (9.1%) of the 44 pigs checked as they entered production in 2001 were found seropositive, all by ELISA. In 2002, five (11.9%) of the 42 pigs checked were found to have anti-*Trichinella* antibodies, three by ELISA and two by western blotting only (see

Table). Overall, 181 pigs were investigated by serology and 36 (19.9%) found seropositive (see Table).

Seroprevalence was found to increase significantly with age, from 16.1% among the animals aged >6-months–<2 years to 18.4% in those aged 2–3 years and 27.3% in the pigs that were >3 years of age (χ^2 for trend; $P<0.01$). Gender also appeared to have an effect on seroprevalence, with 15.3% of the female pigs investigated and 47.8% of the male found seropositive ($P<0.001$).

When, in 2002, 35 pigs that had been found seronegative 6 months earlier were re-tested, three (8.6%) were found seropositive by ELISA and/or western blotting. Two pigs that had been found seropositive in the initial screening were still seropositive, however, when re-tested 18 months (one pig) or 23 months (one pig) later.

Worryingly, at least one seropositive pig was found in each of 15 breeding pens in the study area — 14 (50%) of the pens in urban or peri-urban areas and one (25%) of those in rural areas. The small sample size meant that the likelihood of finding a seropositive pig in an urban/peri-urban breeding pen was not significantly higher than that of finding such a pig in a rural pen (odds ratio = 3.2; 95% confidence interval = 0.23–92; $P>0.32$).

PREVALENCE DETERMINED BY MUSCLE DIGESTION
Of the samples of muscle collected from 55 pigs slaughtered in 2000 (before the serological investigations had begun), four (7.3%) were found to contain *Trichinella* larvae when digested.

In 2001, 105 pigs were checked by digestion and five (4.8%) were found infected. Twenty-three of the 105 pigs investigated by digestion in 2001 were old enough to have been tested in an earlier serological survey. Although all 23 had been found seronegative, three of them were found positive by digestion. The other 82 pigs checked at slaughter in 2001 (including the other two animals found positive by digestion) were piglets aged <6 months and had not therefore been checked by serology.

Overall, 300 pigs were studied by means of artificial digestion between 2000 and 2002, and 12 (4%) tested positive (see Table).

At the end of 2002, 17 seropositive pigs were killed. When 14 of these pigs were checked by digestion (with ≤125 g pork checked/pig), nine (64.3%) were found infected with *Trichinella* larvae, the parasite burden varying from 0.007–282 larvae/g muscle.

Rodents
Overall 26 rodents were caught, the level of trapping success (measured as the number of rodents caught/100 trap-nights) being higher in the pig-breeding pens than in the rubbish dump (2.9 *v.* 1.6) and higher in the pens associated with the two human cases seen in 2000 (the rodent collections being made after all the pigs from these two pens had been culled by order of the National

TABLE. *Detection of* Trichinella *infection in pigs from the Sierra Grande area of the Argentinian province of Río Negro, 2000–2002*

Year of investigation	Serology		Muscle digestion	
	No. of pigs investigated	No. and (%) found positive	No. of pigs investigated	No. and (%) found positive
2000	95	27(28.4)	55	4(7.3)
2001	44	4(9.1)	105	5(4.8)
2002	42	5(11.9)	140	3(2.1)
All three	181	36(19.9)	300	12(4.0)

Animal Health Service) than in the other pens that still held pigs (5.7 *v.* 2.3).

Four (15.4%) of the rodents — three (43%) of the seven caught in the dump and one (5%) of the 19 from the pig-breeding pens — were found infected with *Trichinella* larvae.

Identification of *Trichinella* Isolates

Two isolates of muscular larvae from pigs slaughtered in 2002 and one from a rodent (*Mus musculus*) were investigated by nested PCR. All three isolates gave a banding pattern characteristic of *T. spiralis* (data not shown).

Human Infection

Twelve (10.7%) of the 112 patients tested in ELISA were found seropositive for *Trichinella* infection.

DISCUSSION

Although there are no large-scale producers of pork in Río Negro province, there have been recurrent, small outbreaks of porcine and human trichinellosis in the region for many years. The porcine disease is considered endemic in Sierra Grande, which appears to be the worst affected area in the province. Among the risk factors that probably contribute to this situation are the substandard facilities for pig rearing [staff of the local veterinary service only consider one of the 27 breeding pens in the Sierra Grande area to have 'adequate' facilities (unpubl. obs.)], the feeding of pigs with swill collected from the municipal rubbish dump, and the subsistence economy of the area, with its associated low standards of sanitation, hygiene and housing. It is clear from the present results that, in 2000–2002, the seroprevalence of *Trichinella* infection in the pigs of this area (19.9%) was almost as high as the prevalence recorded, by trichinoscopy, among the pigs slaughtered in the mass cull of 1984 (22%). Although

the ELISA and blotting assays used in the present study are probably more sensitive methods of detecting *Trichinella* infection than either trichinoscopy or muscle digestion, the seroprevalences and seroconversions recorded in 2000–2002 are worrying. Similarly, even though the sample was small, the prevalence recorded in the rodents (15.4%) is also cause for concern, indicating that, as observed in other regions of the world (Loufty *et al.*, 1999; Theodoropoulos *et al.*, 2003), a 'reservoir' of *Trichinella* may be maintained by horizontal transmission among local rats and mice, even in the absence of pigs. Rodent densities in the study area (as indicated by the 2.7 rodents caught/100 trap-nights) appear much lower than those recorded, by Larrieu *et al.* (2002), elsewhere in Río Negro province (12–35 rodents/100 trap-nights). The size of the rodent population in the Sierra Grande area is probably severely limited by the scarce water and food supplies.

In the present study, the age-related changes seen in the seroprevalences among the pigs, the seroconversion (i.e. from seronegative to seropositive) of several pigs over just 6 months, the detection of seropositive pigs in more than 50% of the local breeding pens and in urban, peri-urban and rural settings indicate wide-spread transmission at substantial levels. In terms of the local epidemiology of porcine trichinellosis, the high prevalence of infection recorded among the (small) sample of rodents from the municipal dump may be very significant.

There is a small possibility that both seropositive pigs that were found seropositive again when re-checked after 18 and 23 months had been seronegative and re-infected between the two periods of serology. It seems much more likely, however, that, once seropositive, a pig does not become seronegative for at least a few years.

Given the relatively high seroprevalence of infection in the local pigs, it is perhaps surprising that more cases of human trichinellosis are not detected. The results of the human serology in the present study

(although not based on an unbiased, cross-sectional sample) indicate that human infection with *Trichinella* is much more common (seroprevalence = 10.7%) than symptomatic human trichinellosis (no local cases detected during 2001–2002). Many of the local residents also rarely if ever eat pork, since almost all of the meat produced in the small number of pig-breeding pens is eaten by the pig owners or their families. To minimize the risk of human infection, perhaps sera from all pigs that are coming up to slaughter should be tested in ELISA or western blots (which seem more sensitive than muscle digestion and can easily be used to investigate many samples at one time), so that the seropositives can be discarded (Geerts *et al.*, 2002). Such screening could form the basis of an effective system of surveillance and control although, given the rodent infections, such a system would probably have to be maintained indefinitely.

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