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Diagnosis of cystic echinococcosis on sheep farms in the south of Argentina: areas with a control program

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Abstract

In 2000 Guarnera et al. proposed using ELISA in canine faeces collected from the ground to detect dogs infected with *Echinococcus granulosus*, thus determining sheep farms with active transmission. The objective was to evaluate the prevalence of *E. granulosus* infection in sheep farms of the Patagonia. Sheep farms were randomly selected in the Provinces of Río Negro, Chubut, Neuquén, Santa Cruz and Tierra del Fuego (areas with control programs) and La Pampa (comparison area). From one to three samples of fecal matter were obtained for each sheep farm, which were processed by means of copro-ELISA test with confirmation of positive samples by copro-Western blot. A total of 1042 samples were obtained from 352 sheep farms, 26 (7.3%) proving positive. Of these 5 (6.3%) were from La Pampa, 9 (13.8%) from Neuquén, 4 (4.7%) from Río Negro, 2 (2.9%) from Chubut, 1 (5.9%) from Santa Cruz and 5 (13.9%) from Tierra del Fuego. The identification of parasitized dogs is an essential activity upon which rests the strategy of control and surveillance. Arecoline tests or coproantigen test with fecal matter obtained directly from the dog contribute information on individual prevalence, while the use of coproantigens detected in ground-collected samples transfers the dog unit of observation to units of greater epidemiological value. In the present experience, the technique employed seems promising for its application in systems of epidemiological surveillance of cystic echinococcosis and

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in drawing a baseline on which to measure the progress of control programs in the Argentine Patagonia in subsequent years.

Keywords: Echinococcosis; Epidemiological surveillance; Coproantigens

1. Introduction

Cystic Echinococcosis, produced by *Echinococcus granulosus*, is an endemic zoonosis in the provinces of southern Argentina. Hence, as from 1980 the Public Health services have maintained control programs based on periodic deparasitization of urban and rural dogs with praziquantel (Larrieu et al., 1989, 2002).

These control programs included surveillance systems of canine infection by means of oral administration to a percentage of dogs of the taeniafuge arecoline hydrobromide, with identification of mature *E. granulosus* in eliminated faeces (Schantz, 1973). The drug has been applied to dogs in certain geographical sites, with the proprietors' voluntary attendance. The information thus obtained is the prevalence of canine infection and it is expressed, in surveillance systems, as the percentage of infected dogs (Larrieu et al., 1989).

This technique however presents limitations such as limited sensitivity since not all dogs respond to the purge and not all parasitized dogs eliminate *E. granulosus*. The predictive value of the test therefore diminishes as the percentage of infected dogs decreases (as a result of the application of control measures); it may generate environmental contamination due to the elimination of highly infective eggs into the milieu and it exerts marked secondary actions on treated dogs (Schantz, 1973; Lopera et al., 2003).

Over the last few years, immunodiagnostic techniques based on the identification of parasitic antigens in recently emitted fecal matter, or fecal matter extracted directly from the rectal of the dog (Allan et al., 1992; Deplazes et al., 1992; Craig, 1997), have been developed as alternatives for the surveillance of canine echinococcosis. Copro-ELISA test, for example, has been used by control programs in Cyprus, Spain and Peru (Christofi et al., 2002; Deplazes et al., 1994; Moro et al., 1999), data being expressed in surveillance systems as percentages of infected dogs.

Comparative results of the use of arecoline test and of copro-ELISA test have been published. The prevalences found during field studies were greater with copro-ELISA test than with arecoline test in Viracocha, Perú (46% and 32%, respectively, Moro et al., 1999) and in Tupac Amaru, Perú, (82% and 34%, respectively, Lopera et al., 2003), while there was a minimal difference in La Paloma, Uruguay (22.7% and 23.6%, respectively, Craig et al., 1995)

Using the arecoline test as standard test, the copro-ELISA test gave 95% of specificity (Lopera et al., 2003) and 50%, 76.9% and 88% of sensitivity (Moro et al., 1999; Craig et al., 1995; Lopera et al., 2003), although these results must be interpreted with precaution due to the arecoline test with 70% of sensitivity, for it limitates the identification of all the parasitized dogs (Schanz, 1973).

Using necropsy as standard test, the incubation period for coproantigen detection in experimental infected animals was between 10 and 30 days post infection. Likewise Deplazes et al. (1992, 1994) and El-Shelabi et al. (2000), determined a global sensitivity of 56%, 63% and 61.5% and a sensitivity of 97%, 98% and 91%, respectively. Either the incubation period or the sensitivity results were in proportion to the amount of parasites in the dog's intestine. (100% of sensitivity with more than 100 taenias per dog).

A new system of canine echinococcosis surveillance based on the use of the diagnostic complex copro-ELISA + copro-Western blot using dry faeces collected from the environment, without identification of the emitting dog and without the application of taeniafuges, has been recently proposed (Guarnera et al., 2000).

In carriers dogs of *E. granulosus* the sensitivity and specificity of this system is 100%, while the prevalence found by the complex copro-ELISA + copro-Western blot is superior than the one obtained by the arecoline test (45.4% and 33.3% respectively, Guarnera et al., 2000).

The aim of the present work is to evaluate the percentage of sheep farms infected with *E. granulosus* in provinces of southern Argentina, in order to assess the results of over 20 years of control programs and to establish a new baseline, appropriately standardized, on which to measure the progresses in control during coming years.

2. Materials and methods

2.1. Work area

Control area: five provinces located in the region of the Argentine Patagonia, four continental (Neuquén, Río Negro, Chubut and Santa Cruz) and one insular (Tierra del Fuego), divided into 53 departments, and extending from the south of rivers Colorado and Barrancas (36°45′ South Latitude) down to the cape of Hornos (56°00′ South Latitude), for a total area of 787,054 km^2. Historically, this region has presented high rate of cystic echinococcosis in human populations and maintains systematic control programs (Larrieu et al., 1989, 2002).

From the phytogeographic point of view, this area comprises two distinct regions: (1) the Patagonian steppe with its succession of stepwise plateaus, scarce rainfall (less than 300 mm annually) and extreme temperatures; and (2) the region of the Patagonian Andean forests, located in mountain ranges, characterized by high precipitation (over 2500 mm annually) and low winter temperatures.

The main activity is represented by sheep production for wool (8,304,504 animals) distributed among approximately 7500 agricultural establishments.

Comparison area: Province of La Pampa located to the north of the Patagonian region in the center of the country. It has a surface of 143,440 km^2, divided into 22 departments. It present sporadic appearance of cystic echinococcosis cases in human populations and does not maintain systematic control programs (Lamberti et al., 1999; Larrieu et al., 1996).

From the phytogeographic standpoint, it includes the last features of the humid pampas to the northwest and the distinctive signs of the Patagonia in the remainder of the territory, the main pastoral activity being the breeding of bovines and agriculture. La producción ovina se limita al noroeste de la Provincia y en los establecimientos productores de bovinos como producción secundaria para el abastecimiento de carne (Map 1).

2.2. Sample

The size of the sample (‘‘*n*’’: number of sheep farms involved) was determined with software Epinfo 6.0 with the following parameters: 95% level of trust, 20% of error margin and an expected prevalence of 10% in continental areas and of 1% in the insular one. The selection of the sheep farms to be surveyed was randomly made for each province on the basis of maps or Animal Sanitary Service registrations of sheep farms, according to the availability in each jurisdiction. To do the sampling, each farm was numbered and sorted ‘‘*n*’’ those which were involved to do the study.

Collection was standardized as follows: (1) recently emitted or dry samples of canine fecal matter for each sheep farm up to 500 heads, (2) samples for farms with 500 to 1000 heads and (3) samples for farms with more than 1000 heads. An additional sample was incorporated for each sheep farms with more than three separated keeper’s house.

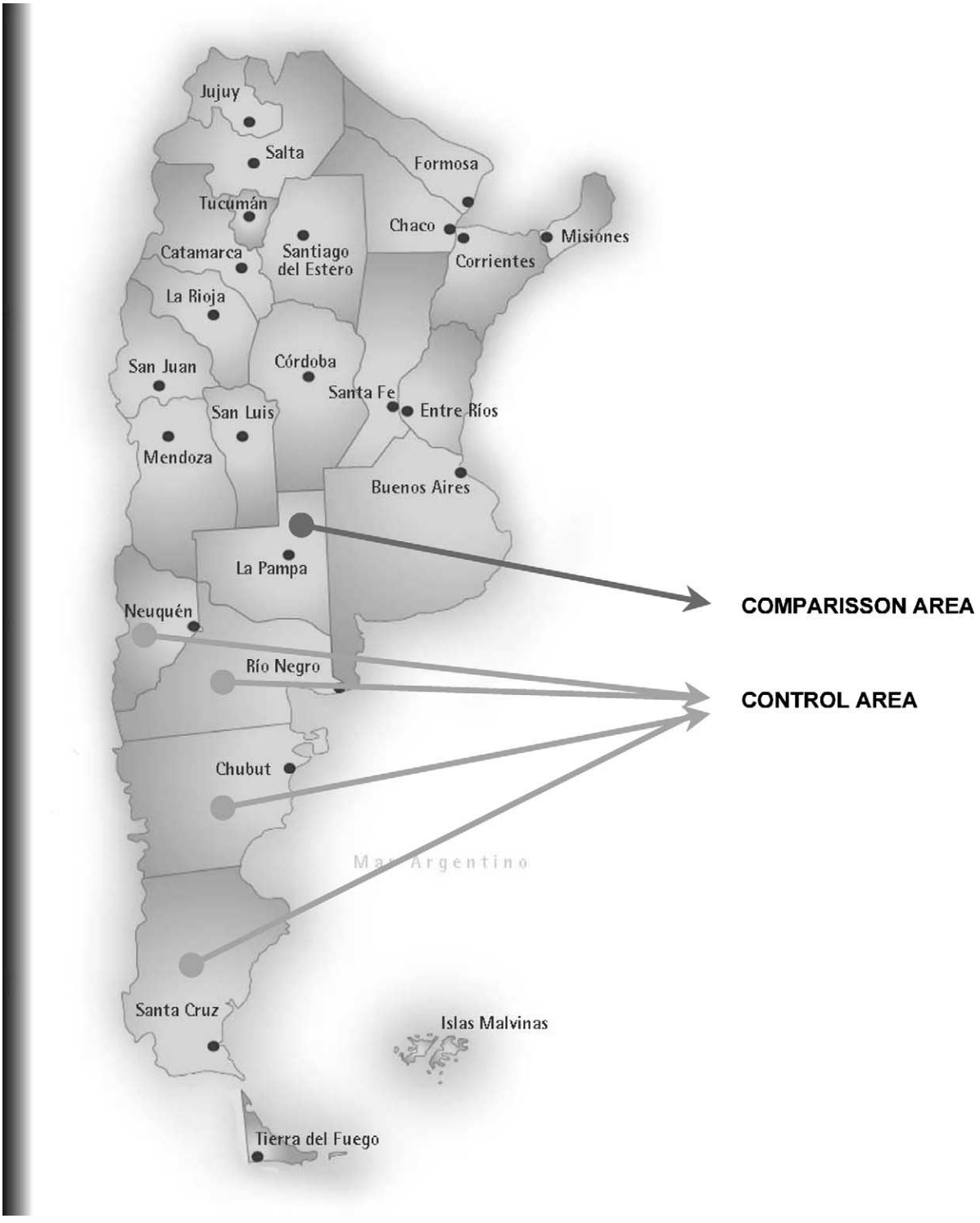
Samples were taken either near the farmyard or near the keeper’s house, to ensure that the faeces belong to dogs and not to other carnivorous.

Areas of fiscal lands and indigenous reservations were assimilated to the concept of a sheep establishment, although the infection prevalence was considered separately for each animal proprietor.

Obtained by veterinarians involved in control programs, samples were placed in wide-mouthed plastic flask with a hermetic seal (biological sample collector type, capacity 120 cc) and preserved in the freezer or in a cool place until their shipment to the Laboratory of Environmental Health in San Carlos de Bariloche, following the general norms of biological material transport. Once received in the laboratory, samples were frozen at −80 °C during 48 h or at −20 °C during 10 days to inactivate *E. granulosus* eggs.

2.3. Laboratory

The dog faeces samples were mixed with same volume of PBS–Tween 20 diluted 0.3%, shaken vigorously and left overnight in a fresh place to obtain



Map 1. Argentina and Patagonian region (program area and control area).

a complete hydration of the faeces. Next day the process was repeated again and some parts were dissolved with the help of a glass rod; as a result, the faeces were broken completely and left quietly for 4 h. After that the extraction was done with centrifugation for 30 min at 3500 rpm at room temperature. Then the supernatant was separated and kept at −20 °C or −70 °C until processed.

Copro-ELISA test was performed according to standardized techniques (Allan et al., 1992; Baronet et al., 1994), as follows:

Plates were sensitized with 100 μl of anti-*E. granulosus* of hyperimmune rabbit serum, diluted up to optimal concentration with carbonate/bicarbonate buffer pH 9.6, overnight at 4 °C in a moist chamber. After three washes with PBS–Tween 0.1% for 5 min, plates were blocked with 200 μl of PBS–Tween 0.3% for 1 h at room temperature; they were washed three times again for 5 min, and 50 μl of inactivated foetal-bovine serum and 50 μl of the floating matter of the diluted faeces were added and incubated for 1 h at 37 °C.

They were washed three times more and 100 μl of conjugate anti-*E. granulosus* marked with peroxidase diluted in phosphate-buffered saline (PBS)–Tween 0.1% was added to it. They were incubated for 1 h at 37 °C, and washed three times; 200 μl of ABTS was added (ABTS, citric acid 0.05 M pH 3.5 and hydrogen peroxide dil. 1/16). They were incubated for 10 min at 37 °C and the reaction was stopped with 100 μl of fluorhydric acid 0.1 M pH 3.5. The reaction was read at 405–410 nm in ELISA reader.

The cutoff value was determined by the mean plus three standard deviations of the optical densities of 30 negative fecal matter samples.

In turn, samples classified as positive by copro-ELISA were processed at the Department of Parasitology, National Institute of Infectious Diseases ''Carlos G Malbrán'' by means of the copro-Western blot test (Towbin et al., 1979; Guarnera et al., 2000), as follows.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in reduced conditions on 12% polyacrylamide gel was carried out by the procedure described by Laemmli (1979). Separated proteins were transferred to a nitrocellulose membrane for 1 h at 250 mA (Trans-Blot Cell, Biorad). The nitrocellulose membrane was blocked with 5% skimmed milk (Difco M.R.) in PBS–0.1% Tween 20 (PBST) overnight at 4 °C. After three washes with PBS–Tween, the nitrocellulose filters were incubated with total anti-*E. granulosus* somatic serum diluted 1:30 in PBS–Tween plus 1.5% skimmed milk.

Filters were subsequently incubated with horseradish peroxidase conjugated goat anti-rabbit serum (Sigma M.R.) diluted 1:500 in PBS–Tween plus 1.5% skim milk. Membranes were washed three times with PBS–Tween and immersed in a solution of 3-392 diaminobenzyline (Fluka M.R.). After 5 min the reaction was stopped by washing with abundant water.

Copro-Western blot test was considered positive by the presence of two bands of molecular weights 40 and 45 k Da (Guarnera et al., 2000).

The copro-ELISA + copro-Western blot diagnostic system was considered as indeterminate when copro-ELISA was positive and copro-Western blot negative (Guarnera et al., 2000).

2.4. Interpretation of results

A sheep establishment was considered infected when in the property there was at least one sample of canine fecal matter that proved positive to both copro-ELISA and copro-Western blot.

3. Results

A total of 352 sheep establishments in 24 departments were surveyed, collecting 1042 samples of fecal matter.

As regards the ''dog'' surveillance unit, in the control area 50 samples (6.0%, 95% CI 4.5–7.8%) proved positive to copro-ELISA and were confirmed by copro-Western blot 25 (3.0%, 95% CI 1.9–4.4), while in the comparison area (La Pampa Province) the number of samples positive to copro-ELISA was 8 (3.7%, 95% CI 1.7–7.4), 5 of which (2.3%, 95% CI 0.8–5.6) were confirmed by copro-Western blot. Discrimination by province showed for the control area a range of confirmed samples from 1.1 (Chubut) to 7.0% (Neuquen) (Table 1).

Differences between the control area and the comparison area were not significant (p 0.5; OR 0.8; 95% CI 0.2–2.1).

Table 1
Survey by means of copro-ELISA + copro-Western blot of canine echinococcosis prevalence in sheep establishments in southern Argentina, per province, 2003

Province	Establishments no. surveyed (%)	Sample collected no.	Copro-ELISA + samples no. (%, 95% CI)	Copro-Western blot + samples no. (%, 95% CI)
La Pampa	79 (22.4)	214 (20.5)	8 (3.7, 1.7–7.4)	5 (2.3, 0.8–5.6)
Comparison area	79 (22.4)	214 (20.5)	8 (3.7, 1.7–7.4)	5 (2.3, 0.8–5.6)
Neuquen	65 (18.5)	157 (15.1)	14 (8.9, 5.1–14.7)	11 (7.0, 3.7–12.4)
Rio Negro	86 (24.4)	257 (24.7)	18 (7.0, 4.3–11.0)	5 (1.9, 0.6–4.6)
Chubut	69 (19.6)	175 (16.8)	5 (2.9, 1.6–8.2)	2 (1.1, 0.1–4.4)
Santa Cruz	17 (4.8)	59 (5.7)	2 (3.4, 0.8–12.7)	1 (1.7, 0.0–10.0)
Tierra del Fuego	36 (10.2)	180 (17.3)	11 (6.1, 3.2–10.9)	6 (3.3, 1.3–7.4)
Control area	273 (77.6)	828 (79.5)	50 (6.0, 4.5–7.8)	25 (3.0, 1.9–4.4)
Total	352 (100)	1042 (100)	58 (5.6, 4.3–7.2)	30 (2.9, 2.0–4.1)

Table 2
Results of confirmation copro-Western blot test to determine the presence of cystic echinococcosis in sheep establishments in southern Argentina, per province, 2003

Province	Copro-ELISA + establishments no. (%, 95% CI)	Copro-Western blot + establishments no. (%, 95% CI)
La Pampa	7 (8.8, 3.8–17.8)	5 (6.3, 2.1–14.3)
Comparison area	7 (8.8, 3.8–17.8)	5 (6.3, 2.1–14.3)
Neuquen	12 (18.5, 3.6–50.9)	9 (13.8, 1.1–52.0)
Rio Negio	13 (15.1, 8.5–24.8)	4 (4.7, 1.5–12.2)
Chubut	5 (7.2. 2.6–16.7)	2 (2.9, 0.5–11.0)
Santa Cruz	2 (11.8, 2.0–37.7)	1 (5.9, 0.1–29.6)
Tierra del Fuego	5 (13.9, 5.2–30.3)	5 (13.9, 5.2–30.3)
Control area	37 (13.6, 9.3–17.8)	21 (7.7, 4.4–11.0)
Total	44 (12.5, 9.3–16.5)	26 (7.3. 4.9–10.6)

As regards the ‘‘sheep establishment’’ surveillance unit, in the control area there were 37 samples (13.6%) positive to copro-ELISA, 21 of which (7.7%) were confirmed by copro-Western blot, while in the comparison area (La Pampa Province) the number of infected establishments by copro-ELISA was 7 (8.8%), 5 of which (6.3%) was confirmed by copro-Western blot. Discrimination by province showed for the area control a range of infected sheep establishments from 2.9% (Chubut) to 13.8% (Neuquen) (Table 2).

Differences between the control area and the comparison area were not significant (p 0.4; OR 0.7; 95% CI 0.2–2.0).

In the control area, 57.9% of surveyed departments were free of infection, while in 42.1% infected sheep establishments were located. In the comparison area, on the other hand, 60% of surveyed departments were free of infection, while 40% presented infected sheep establishments.

An analysis of the departments where infected sheep establishments were identified showed a range from 3.7% (Senguer, Chubut) to 28.6% (Puelen, La Pampa) sheep establishments infected by department (Table 3).

From a point of view of geographical distribution, sheep establishments were located in the north end of the control area (Catrilo, La Pampa Province); to the west, in mountain range departments bordering with the Republic of Chile (Senguer, Chubut; Lago Argentino, Santa Cruz); to the east, in departments bordering with the Atlantic ocean (Escalante, Chubut) and in central regions of the Patagonia (Zapala, Neuquén; 25 de Mayo, Río Negro).

Table 3
Results of confirmation copro-Western blot test to determine the presence of cystic echinococcosis in sheep establishments in southern Argentina, per department, 2003

Province	Department	Surveyed establishments no.	Copro-Western blot + establishments no. (%, 95% CI)
La Pampa	Guatrache	20	
	Catrilo	23	1 (4.3, 0.2–23.8)
	Realico	5	
	Puelen	14	4 (28.6, 9.5–58)
	Atreuco	17	
Neuquen	Catan Lil	22	5 (22.7, 8.6–45.7)
	Zapala	35	
	Huiliches	8	
Rio Negro	A. Alsina	5	
	Bariloche	14	
	9 de Julio	21	
	25 de Mayo	29	
	El Cuy	1	
	Gral Roca	3	
	Pilcaniyeu	12	1 (8.3, 0.4–40.2)
	San Antonio	1	
Chubut	Escalante	11	1 (9.1, 0.4–42.9)
	Ameghino	4	
	Sarmiento	27	
	Senguer	27	1 (3.7, 0.1–20.8)
Santa Cruz	Lago Argentino	7	1 (14.3, 0.1–58.0)
	Guer Aike	2	
	Rio Chico	8	
Tierra del Fuego	Rio Grande	36	5 (13.9, 5.2–30.3)
Total		352	26 (7.3, 4.9–10.6)

4. Discussion

The identification of canine *E. granulosus* carriers on the part of control programs in the Argentine Patagonia has been a high-priority activity included in surveillance systems, using the arecoline hydrobromide test. The latter has contributed valuable information on individual prevalence during many years, but the decrease in prevalence figures taking place in the region currently limits the interpretation of results due to its low predictive value (Larrieu et al., 1989, 1999).

ELISA test is a simple and economic technique, ideal for screening test. On the contrary Western blot requires a sophisticated equipment and a well-trained operator yet it is an election test for confirmation. (Verastegui et al., 1992).

The use of the copro-ELISA + copro-Western blot system for the identification of antigens in ground-collected faeces (Guarnera et al., 2000) has transferred the ‘‘parasitized dog’’ observation unit to observation units of greater value for epidemiological interpretation, such as ‘‘infected sheep establishment’’ where recent transmission of cystic echinococcosis is identified, a vital factor for the instrumentation of control actions specifically directed with a risk focus, as for example increased deparasitization frequencies of establishment dogs or measures of a legal nature such as closures and fines.

The simplicity and economy of sample collection and preservation systems, the possibility to obtain specimens in geographical areas of difficult access, the possibility that sampling is carried out by sanitary agents of primary health attention

programs and the acceptable sensitivity and specificity of the method jointly grant to copro-ELISA + copro-Western blot a high epidemiological value as a surveillance system of cystic echinococcosis.

The design applied which allows to take several faeces samples from most of the sheep farmers to evaluate transmission and classify the farm as *E. granulosus* positive minimize the sensitivity limit of the copro-ELISA test because it increases the chances to find at least one infected dog.

Collaterally, for control programs the information contributed exclusively by copro-ELISA may prove of interest, whereas in sheep establishments positive to this technique and negative to copro-Western blot, the presence of dogs parasitized by other taenias (*Taenia ovis*, *Taenia hydatigena*) may be suspected, indicative of feeding dogs with raw viscera or of insufficient deparasitation coverage, in both cases providing an epidemiological alert of interest.

Results achieved allow it to be inferred that in spite of the control effort applied over the last decades (Larrieu et al., 2002; Zanini, 2002), cystic echinococcosis is still present throughout the territory of the Argentine Patagonia.

However, on 57.9% of its surface infected sheep establishments are no longer identified, thus approaching the rate of the comparison area, which expresses a striking success in the control of the disease in rural areas where climatic and geographic factors limit control measures during a large part of the year. As an example, infection prevalence rates in dogs at the beginning of control activities were 28.2% in Neuquén and 41.5% in Río Negro (Larrieu et al., 2002). In fact, in the present experience there have been no significant differences in infection by *E. granulosus* between comparison and control areas.

The percentage of samples positive to the copro-ELISA + copro-Western blot complex (2.9%) is compatible with the current canine echinococcosis prevalence, determined both in the control area by means of dog dosage with arecoline hydrobromide, for example 2.3% in Río Negro and 2.5% in Tierra del Fuego (Larrieu et al., 2002; Zanini, 2002) and in the comparison area, with 2.6% of infected dogs and 8.3% of sheep establishments with infected dogs in La Pampa Province, proving similar to the figures identified by means of coproantigens in other control program, as in Cyprus, with a prevalence ranging from 1.1% to 4.9% (Christofi et al., 2002).

In the present experience, the copro-ELISA + copro-Western blot system has been promising for its application in epidemiological surveillance systems for cystic echinococcosis and for drawing a baseline on which to measure progress in control programs in subsequent years.

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