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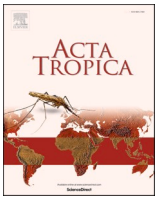

Echinococcus granulosus sensu stricto and *E. canadensis* are distributed in livestock of highly endemic area in the Peruvian highlands

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ABSTRACT

The prevalence of cystic echinococcosis is high in many livestock areas of Peru, where intermediate hosts such as sheep, cattle, and South American camelids can be infected. Several species of *E. granulosus* have been described in relation to its genetic diversity and distribution. The aim of this study was to determine the species of *E. granulosus sensu lato (s.l.)* metacestodes collected from sheep, cattle, swine and camelids at different localities in the department of Puno, in the southern highlands of Peru. One hundred and fifty-two echinococcal cysts were collected from 10 different locations. *E. granulosus s.l.* species were determined by amplification of the Internal transcribed spacer 1 of the ribosomal DNA using a Nested PCR-RFLP technique. The cytochrome C oxidase 1 gene (450 bp) was also amplified and sequenced in samples with different RFLP patterns. Cysts samples were collected from sheep (39.5%), cattle (32.9%), pigs (15.8%) and alpacas/llamas (11.8%). *E. granulosus sensu stricto* (G1 genotype) was mainly identified in all animal hosts, while, the *E. canadensis* (G7) was only identified in cysts from pigs and alpacas. This is the first report of *E. granulosus sensu stricto and E. canadensis* in llamas and alpacas, respectively. Knowledge of species and molecular epidemiology of *E. granulosus s.l.* in endemic areas in Peru may help to evaluate preventive programs, understand disease transmission, as well as improve vaccine and chemotherapy effectiveness.

1. Introduction

Cystic echinococcosis (CE) is a parasitic zoonosis produced by the larval stage of the taeniid cestode *Echinococcus granulosus sensu lato* (*s.l.*). This parasite fulfills its biological cycle among domestic dogs or some wild canids as definitive hosts and livestock animals as intermediate hosts (Bowles and McManus, 1993b; Eckert et al., 2001). Humans may also act as intermediate hosts after accidental ingestion of parasite eggs. The main factors for the transmission and persistence of the infection include the close coexistence of animals or humans with dogs, deficient hygienic-sanitary conditions, cultural customs and low socio-economic status (Apt et al., 2000).

Human CE has a worldwide distribution, occurring in many parts of South America, including Argentina, Peru, Bolivia, Uruguay, Chile and the southern part of Brazil (Cucher et al., 2016; Pavletic et al., 2017). The Andean locations of the central and southern highlands of Peru have the conditions to maintain the biological cycle of the parasite; these areas are endemic for animal (sheep and cattle) and human CE (Moro et al., 2004; Almeida et al., 2007; Gavidia et al., 2008). The highest incidences of human CE are distributed to the south and center regions of Peru, with 14 to 43 CE cases/100000 inhabitants reported in Huancavelica, Junín, Pasco and Puno (Cabrera, 2007). Similarly, 73% of CE cases among livestock have been described in the same regions. The intermediate hosts of *E. granulosus* are mainly sheep, cattle or pigs (Núñez et al., 2003). Puno is considered a mixed-breeding livestock area and is endemic for CE in the Andean region of Peru (Leo-Velarde and Quiroz, 2004).

The use of molecular techniques allows the discrimination of

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recognized species within the *Echinococcus* genus. *E. granulosus* is genetically diverse, based on the analysis of nuclear or mitochondrial DNA. This diversity is reflected in the phenotypic characteristics of the parasite in relation to its life cycle, development, control and treatment (Bowles and McManus, 1993b). Based on these analyzes, genotypes (G1-10) and species: *E. granulosus sensu stricto* (*s.s.*), *E. canadensis, E. ortleppi* (causative agents of human CE) and *E. equinus, E. felidis* have been described (McManus, 2013; Chaâbane-Banaoues et al., 2016; Cucher et al., 2016). In South America the presence of different species of *E. granulosus* has been described; *E. granulosus s.s.* (G1) in sheep, *E. ortleppi* (G5) in cattle, *E. canadensis* (G6) in goats and pigs (G7) (Bowles and McManus, 1993a; Cucher et al., 2016). In Peru, only two species have been reported in a few studies and samples; mainly *E. granulosus s.s.* (G1) in sheep, cattle and humans, *E. canadensis* (G7) in pigs and goats (G6) (Moro et al., 2009; Sánchez et al., 2010).

Currently there is a paucity of information about the distribution of circulating *E. granulosus s.l.* in all livestock species within CE endemic regions in Peru. The aim of this study was to determine the species of *E. granulosus s.l.* present in echinococcal cysts from sheep, cattle, pigs, alpacas and llamas at different locations in the department of Puno (Southern Highlands), using PCR-RFLP analysis targeting the Internal transcribed spacer (ITS) 1 segment of ribosomal (r) DNA and sequencing of the cytochrome C oxidase subunit 1 (*cox1*) gene.

2. Materials and methods

2.1. Study area

Cyst samples were collected from 10 provinces in the department of Puno (Fig. 1) Puno is located in the southeast of Peru at 3800 m.a.s.l, with a density of 17.5 inhabitants/km^2. The livestock economic activity is based on mixed breeding of sheep, cattle, pigs, alpacas and llamas.

2.2. Sampling collection

One hundred fifty-two samples of fertile echinococcal cysts were collected from the lung and liver of sheep, cattle, pigs, alpacas and llamas. Samples were collected from animals slaughtered in non-authorized centers (house backyard) by farmers at different locations across the provinces. Animal species and age (estimated by teeth eruption), location and viability of the cysts was also recorded. Cysts were aseptically incised for the extraction of *E. granulosus s.l.* protoscolices, the liquid content was kept at room temperature (~10°C) for 5 minutes and the supernatant was removed and discarded. Viability of protoscolices was evaluated by 0.1% eosin staining and direct observation under a light microscope (Himonas et al., 1994). Protoscolices were preserved in absolute ethanol for transportation and stored at -20°C, until use.

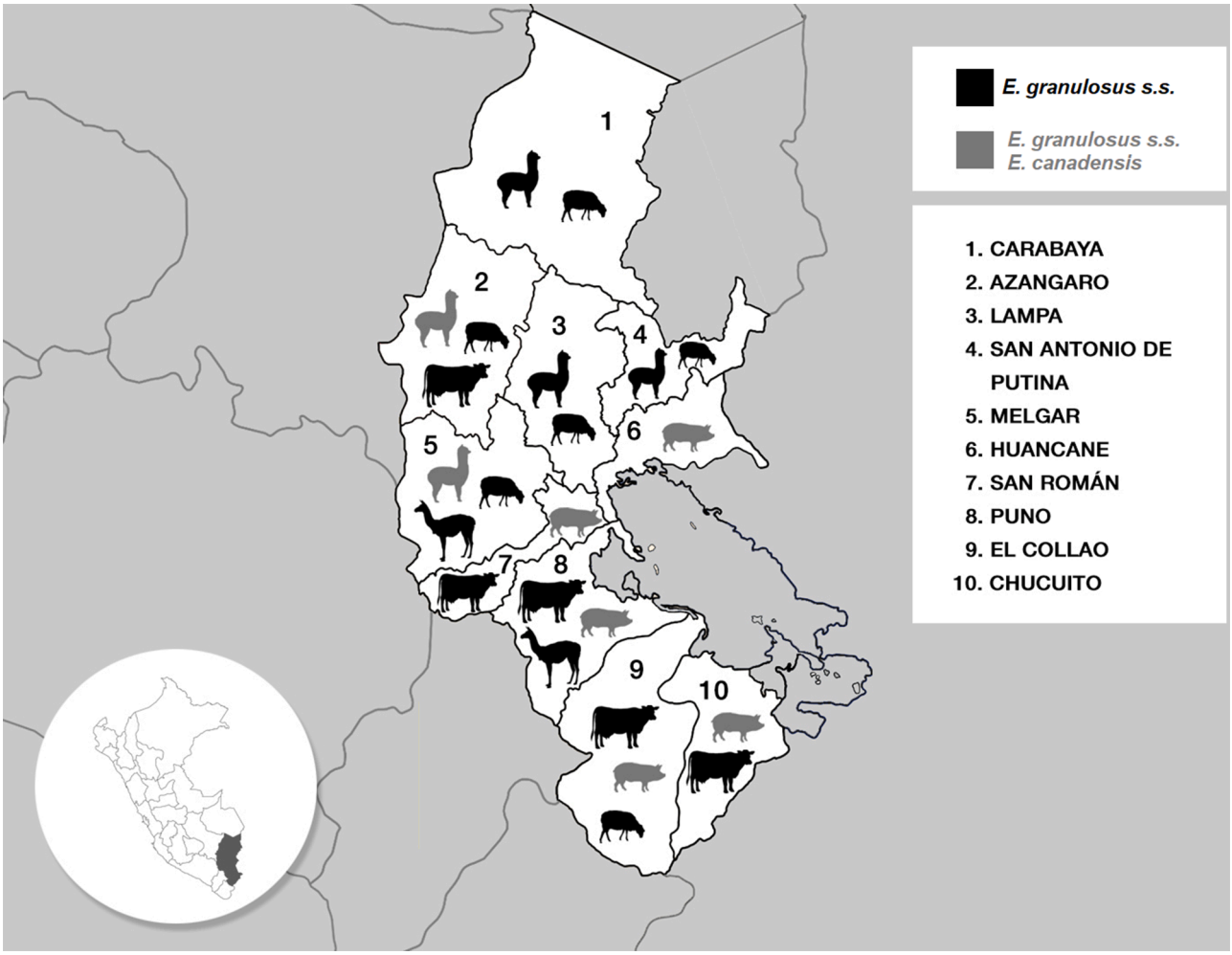

Fig. 1. Geographic distribution of *E. granulosus s.l.* according to livestock species and locations in Puno, Peru.

2.3. DNA isolation

The DNA of each cyst was extracted from protoscolices using the following protocol: protoscolices were washed twice by centrifugation at 1400 x g for 5 min with TE buffer (10 mM HCl and 1 mM EDTA). The pellet containing the cells was lysed with 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 1% SDS, 200 μg/ml of proteinase K and incubated at 56°C overnight. DNA isolation was performed with a modified phenol chloroform method (Sambrook and Russell, 2006). Isolated DNA was resuspended in TE buffer and stored at -20°C in aliquots until use. The concentration and quality of DNA was determined spectrophotometrically (NanoDrop 2000/2000c, Thermo Scientific, USA).

2.4. Genotyping

2.4.1. Nested-PCR RFLP

The ITS1 segment of the rDNA 18S and 5.8S genes of *E. granulosus s.l.* was amplified using a nested-PCR (Bowles and McManus, 1993a-b). The outer PCR targeted an 1800 bp fragment and was carried out using primers 5′-CCAAACTTGATCATTTAGAGGAAG-3′ (forward) and 5′-TATGGGCCAAATTCACTCATTACC-3′ (reverse). PCR reactions contained 40 ng of DNA template, 1X buffer (20 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.25 μM of each primer, and 0.1 U Taq DNA Polymerase (Invitrogen, USA), in a final volume of 20 μl. The cycling conditions consisted of one step of 94°C for 5 min and 30 cycles, each of 94°C for 45 s, 55°C for 90 s and 72°C for 120 s.

The internal-PCR targeted a 1000 – 1100 bp fragment and was carried out using primers 5′-GTCGTAACAAGGTTTCCGTAGG-3′ (forward) and 5′-TAGATAGTGTCGTCGTCGTCGTCGA -3′ (reverse). The master mix was similar to the one used in the external PCR, except that 1 μl of the external PCR product was used as the template and the reaction was carried out in a final volume of 60 μl. The cycling conditions consisted of 35 cycles, each cycle of 94°C for 45 s, 61°C for 45 s and 72°C for 45 s.

2.4.2. Restriction enzyme analysis and cox1 sequencing

A total of 12.5 μl of the internal PCR product was digested using 5 U of restriction enzymes (MspI, RsaI, AluI and DdeI) as described by the manufacturer (New Englands Biolabs, UK) in a final volume of 15 μl. Samples were incubated at 37°C for 4 hours and stopped at 65°C for 20 min. The digested products were electrophoresed in a 2% agarose gel (Invitrogen, USA) stained with 0.5 μg/μl of ethidium bromide and visualized in a UV transilluminator/photo documenter (Sharbatkhori et al., 2010).

Eight random cysts from each different host species (different band patterns among RFLP positive samples) were sequenced. The mitochondrial *cox1* gene fragment was amplified by conventional PCR targeting a fragment of 450 bp using primers 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ (forward) and 5′-TAAAGAAAGAACATAATGAAAATG-3′ (reverse) (Bowles et al., 1994). PCR reactions contained 40 ng of DNA, 1X buffer (20 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.25 μM of each primer and 0.1 U Taq DNA polymerase (Invitrogen, USA), in a final volume of 50 μl. The cycling conditions consisted of 36 cycles, each cycle consisted of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s.

The amplified fragments were commercially sequenced using the Sanger method (Macrogen, Korea). Consensus sequences were aligned using Clustal W with known sequences of *E. granulosus s.s.* (genotypes G1) and *E. canadensis* (G7) obtained from GenBank (accession nos. KX527915; GU233952; SB458678; DQ062858; DQ144022; AY686566; DQ856468). Molecular phylogeny was analyzed using the Neighbor-Joining method (Saitou and Nei, 1987) and MEGA v.6 software (Tamura et al., 2013). Distance matrices were constructed and the phylogenetic tree was generated; bootstrap support for clade topologies was estimated following the generation of 1000 pseudoreplicate datasets.

2.5. Data analysis

E. granulosus s.l. species were presented as proportions according to the different animal hosts, age and location. One-way analysis of variance was used to evaluate the protoscolex viability differences among the animal species for *E. granulosus s.s*. Mann-Whitney tests were used to assess the differences in protoescolex viability for *E. canadensis* (pigs and alpacas). Statistical significance level was set at 0.05; data analysis was performed using Stata 12.0.

3. Results

Cyst samples from sheep, cattle, alpacas and pigs were collected from different provinces, with the exception of the two cysts from llama that were found only in Melgar and Puno (Fig. 1). A total of 39.5% (60/152) cyst samples were obtained from sheep, 32.9% (50/152) from cattle, and the remaining 27.6% (42/152) were obtained from pigs, llamas and alpacas. The majority of the cysts were identified as *E. granulosus s.s* (92.1%, 140/152) and were isolated from all provinces, while only 7.9% (12/152) were identified as *E. canadensis* and they were from Melgar, Azangaro, Collao, Puno, San Román, Huancane and Chucuito (Fig. 1).

The 72.37% (110/152) of cysts were located in lung and liver from sheep and cattle, 13.16% (20/152) were located only in lung from camelids and pigs and 11.84% (18/152) were located only in liver from pigs. All DNA cysts (mean concentration: 113.7 ng/μl and mean 260/280 ratio: 1.88) from different animals such as sheep, cattle, and llama were identified as *E. granulosus s. s.* (G1); however, 9 out of the 24 cyst samples from pigs were identified as *E. canadensis* (G7), and 3 out of the 16 cyst samples from alpaca were also identified as *E. canadensis*. The remaining cyst samples from pigs and alpacas were identified as *E. granulosus s.s*. The average age of animals was 4.9 and the protoscolex viability for *E. granulosus s.s.* was 74.8%, with sheep cysts demonstrating the highest viability (81%). By comparison, the mean protoscolex viability for *E. canadensis* was 65.8%; pig = 63.6% and alpaca cysts = 72.7% (Table 1). No other *E. granulosus s.l.* genotypes were identified among the analyzed samples.

Mean protoscolices viability of *E. granulosus s.s.* was significantly higher than *E. canadensis* ($p=0.002$). *E. granulosus s.s.* in sheep were also more viable than for the other animal hosts ($p<0.001$) with the exception of llamas ($p=0.05$). Viability of cysts from cattle, alpaca and llama was statistically similar to each other but significant different when comparing cattle to pigs ($p<0.001$) and alpacas to pigs ($p=0.01$). Pig and llama cysts had similar viability as well ($p=0.98$). Regarding the *E. canadensis*, there was no statistical difference in viability between alpacas and pigs ($p=0.11$).

E. granulosus s.s. (G1) and *E. canadensis* (G7) were confirmed in randomly selected samples of *E. granulosus s.s.* (n = 6, cattle, llama, sheep, pig and alpaca) and *E. canadensis* (n = 2, alpaca and pig), previously identified by RFLP, by analyzing the partial nucleotide sequence of the *cox1* gene. Sequences were compared with those previously deposited in GenBank (accession nos. KX527915 and MH301022 for G1 and G7, respectively), and they showed more than 99% genetic identity with them. *E. granulosus s.s.* samples clustered with the *E. granulosus s.l.* reference sequence and *E. canadensis* samples in the *E. canadensis* clade (Fig. 2). Within the *E. granulosus s.l.* clade there was evidence for variation among samples collected across Puno. Partial consensus sequences resulted in a mean of 415 nucleotides, the *cox1* gene of *E. granulosus s.s* in llamas and *E. canadensis* in alpacas, described for the first time, were deposited in GenBank under accession nos. MW732663 and MW736596, respectively.

4. Discussion

In this study, the *E. granulosus s.s* (G1) was the most frequently identified in different livestock hosts with high viability. *E. canadensis* (G7) was the other species from only pigs and alpacas with less viability.

Table 1
Distribution of cyst samples and *E. granulosus s.l.* in different host species from the southern highlands of Peru.

Hosts	Age [1] (years)	Infected Organ		*E. granulosus s.l.*				Total (n, %)
				E. granulosus s.s.		*E. canadensis*		
		Lung	Liver	n (%)	Viability (%)	n (%)	Viability [2] (%)	
Sheep	4.4	Yes	Yes	60 (42.9)	81.3 [b]	0 (0)	_	60 (39.5)
Cattle	4.4	Yes	Yes	50 (35.7)	72.8 [b, c]	0 (0)	_	50 (32.9)
Pig	2.5	No	Yes	15 (10.7)	61.3 [b, c]	9 (75)	63.6	24 (15.8)
Alpaca	6.3	Yes	No	13 (9.3)	69.4 [b, c]	3 (25)	72.7	16 (10.5)
Llama	7	Yes	No	2 (1.4)	64.1	0 (0)	_	2 (1.3)
Total	4.9			140	74.8 [a]	12	65.8 [a]	152

[1] Age averaged across each animal species.
[2] Viability was evaluated with 0.1% eosin staining and direct counting expressed in live protoscolices / total protoscolices x 100. Values are included as averages for each host.
[a] Average viability between *Echinococcus* species are statistically different ($p<0.05$).
[b] Average viability of Echinococcus species between hosts were statistically different with respect to sheep ($p<0.001$).
[c] Average viability of *Echinococcus* species between hosts were statistically different with respect to pigs ($p<0.05$).

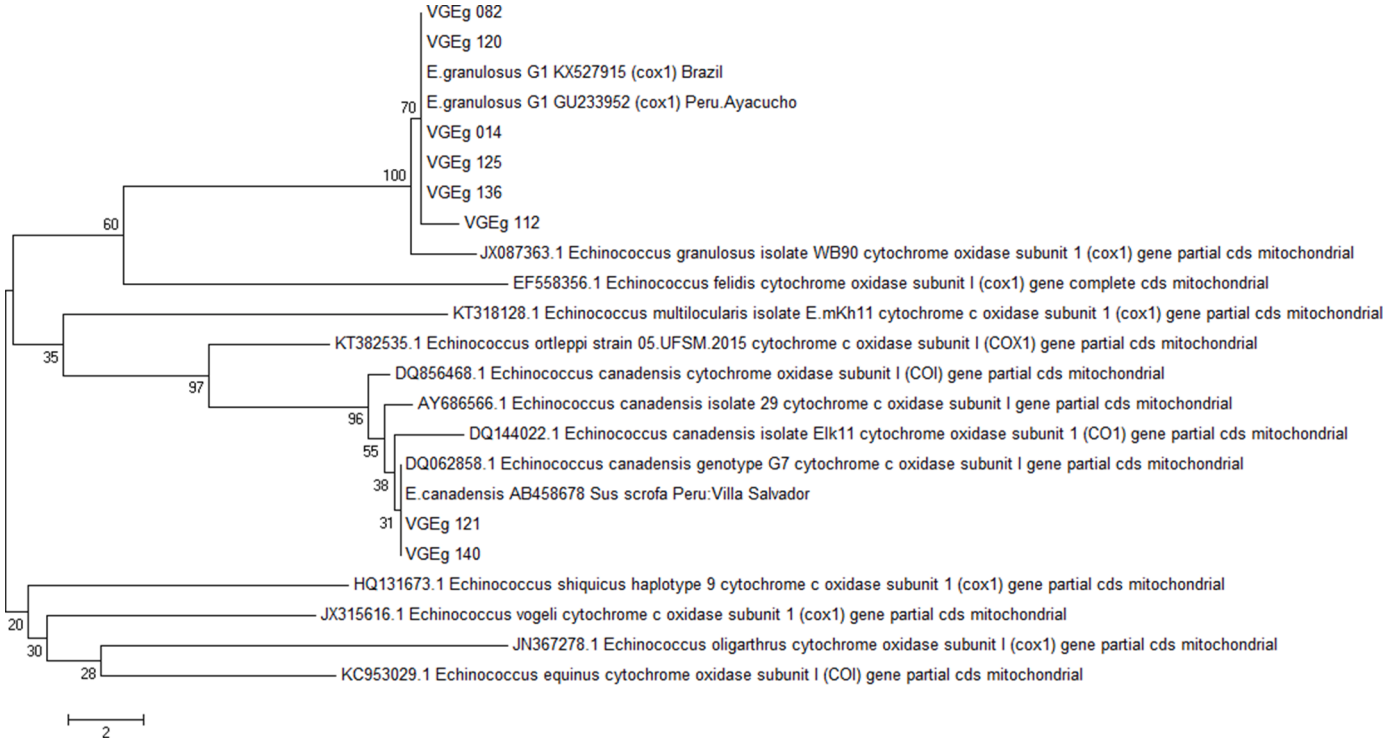

Fig. 2. Evolutionary relationships of the *cox1* gene of *E. granulosus s.l.* cysts. The evolutionary history was inferred using the Neighbor-Joining method, conducted in MEGA6 (Tamura et al., 2013). Sample VGEg 082: cattle; VGEg 120 and 125: llama; VGEg 014: sheep; VGEg 136 and 140: pig; VGEg 112 and 121: alpaca.

To our knowledge, this is the first report of *E. granulosus s.s* in llamas and *E. canadensis* in alpacas worldwide. In Peru, both species of South American camelids number more than 4 million and are used as pack animals, for fiber trading and meat consumption. Camelid rearing is important for the economy of indigenous communities of the Peruvian Andes as it is the only profitable farming alternative in these difficult conditions (Bradford et al., 1989; Leguia, 1991). In this study, llamas were infected with the *E. granulosus s.s*, which is the most ubiquitous and is generally associated with the sheep-dog cycle (Bowles and McManus, 1993b). Previous studies have also identified the G1 genotype in alpacas from the same geographic area (Sánchez et al., 2010). The manner of raising these camelids in the highlands, which is carried out in a mixed way with sheep that roam in the Andes for pasture, has potentially made it suitable for the *E. granulosus s.s.* to adapt over time and these transmission dynamics may contribute to the dispersion of this parasite.

Recently, G7 in pigs and G5 genotype in cattle have been detected in Bolivia (Ali et al., 2020), a neighbouring country to Puno with high trade transit between them. *E. canadensis* in pigs is generally responsible for 67–100% of infections and is more frequent than *E. granulosus s.s.* (Cardona and Carmena, 2013). Interestingly, *E. granulosus s.s.* was less viable in pigs in this study. The difference in viability of protoscolices can be due to some factors, such as temperature and humidity, time of sampling, viability assessment method and variations of the parasite (Shahnazi et al., 2013). Survival and development of cysts depends on factors beyond those relating to host and parasite genetics and location (Rogan et al., 2015).

In Peru, *E. granulosus s.s.* (G1) has been also found in alpacas, sheep, cows, goats and pigs; G6 in goats; and G7 genotype in pigs (Cucher et al., 2016). G7 have been recently detected in other hosts and continents, such as in wild boars in Europe and goats in Asia (Laurimäe et al., 2019; Joanny et al., 2021). In Puno, and other CE endemic locations of Peru, the mixed breeding of different livestock animals may explain the diversity of genotypes found in these intermediate hosts. Frequencies may differ among locations in a same area because of the number of herds and animal species farmed for different livestock purposes.

Fertility of cysts was no registered in this study. *E. granulosus s.s.* (G1)

genotype in sheep produces viable and fertile echinococcal cysts, playing an important role in transmission. In contrast, *E. granulosus s.s.* infections in cattle and pigs are usually infertile, therefore, these hosts may not play as important a role (Thompson, 2017). In humans, this genotype is also responsible for the majority of infections and development of cysts worldwide. Likewise, the cysts generated may be larger than those reported for *E. canadensis* (G6/G7), the second one responsible for CE in humans (Alvarez Rojas et al., 2014).

The lung was the most frequent infected organ recorded in this study, although the liver is the most common site for hematogenous spread of *E. granulosus s.l.* oncospheres after oral infection in humans, but G7 appears to infect preferentially the liver in animals and brain in humans (Cucher et al., 2016). Likewise, contaminted lung or liver from livestock are often voluntary dispensed to shepherd dogs. Regarding genotype variability, canine isolates were assigned as *E. graulosus s.s* or G6 genotypes, and, G7 is also expected to be circulating in dogs of South America (Carmena and Cardona, 2013).

The PCR-RFLP technique can be used to identify different species of *E. granulosus s.l.* Although sequencing is considered the gold standard for genotyping, study findings indicate the PCR-RFLP produces equivalent results. Sequencing is expensive and cannot be used routinely or for epidemiological surveys where many samples need to be processed especially in endemic areas (Chaâbane-Banaoues et al., 2016). A full mitocondrial DNA analysis allows significantly better phylogenetic resolution compared with the *cox1* gene (used in this study), for example the use of mitogenomics can be discover a highly divergent haplotypes G7a and G7b (Laurimäe et al., 2018). The use of a partial and only marker such as *cox 1* gene was a limitation for correct discrimination of genotypes in this study. However, species identification is relevant, because *E. granulosus s.l.* may differ in protein profiles, morphology, carbohydrate and lipid repetoires, metabolic requirements, fertility, intermedite host specificity, pre-patent period, antigenicity, and infectivity and pathogenicity in humans (Cucher et al., 2016); and these differences might have important implications for vaccine-based control programs and diagnosis.

Prevention and control programs have been developed for definitve and intermediate hosts, one of which is the immunization. The EG95 vaccine, first developed against G1 isolates and administrated in trials in the central highlands of Peru, produces high levels of protection in sheep against infection. Variability of the EG95 gene by different genotypes may directly impact the effectiveness of this vaccine, which has been revealed by genotype G6 affecting the control and prevention of CE for various *E. granulosus s.l.* strains (Pan et al., 2017). Similarly, different antigenic sets may be potentially expressed by several genotypes; the extent of this variability can affect the diagnostic performance of antibody-based assays (Siles-Lucas et al., 2017).

5. Conclusion

E. granulosus s.l. in livestock hosts in the South highlands of Peru are restricted to *E. granulosus s.s.* and *E. canadensis*. *E. granulosus s.s.* and *E. canadensis* are reported for this first time in llamas and alpacas, respectively. Further studies are needed to evaluate the role of camelids in CE transmission and to determine the genetic variability of these genotypes in their definitive host in this endemic area.

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CRediT authorship contribution statement

Leny Sanchez: Investigation, Writing – original draft, Project administration. **Holger Mayta:** Investigation, Writing – original draft, Project administration. **Luis M. Jara:** Writing – original draft, Writing – review & editing, Formal analysis, Data curation. **Manuela Verástegui:** Conceptualization, Resources, Supervision. **Robert H. Gilman:** Conceptualization, Resources. **Luis A. Gómez-Puerta:** Resources, Writing – review & editing. **Cesar M. Gavidia:** Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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