Parasitology International 66 (2017) 250–257

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Implementation of new tools in molecular epidemiology studies of *Echinococcus granulosus* sensu lato in South America

Héctor G. Avila [a,b], Guilherme B. Santos [c], Marcela A. Cucher [a], Natalia Macchiaroli [a], Matías G. Pérez [a], Germán Baldi [d], Oscar Jensen [b], Verónica Pérez [e], Raúl López [f], Perla Negro [g], Exequiel Scialfa [h], Arnaldo Zaha [c], Henrique B. Ferreira [c], Mara Rosenzvit [a], Laura Kamenetzky [a,*]

[a] Instituto de Investigaciones en Microbiología y Parasitología Médica, IMPAM (UBA-CONICET), Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, piso 13 (1121), Ciudad Autónoma de Buenos Aires, Argentina
[b] Centro de Investigaciones en Zoonosis, Chacra N°18, Sarmiento, Chubut, Argentina
[c] Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brazil
[d] Estudios Ambientales, Instituto de Matemática Aplicada de San Luis UNSL-CONICET, San Luis, Argentina
[e] División Zoonosis, San Juan:AvLibGral San Martín 750, Centro Cívico 3° piso Núcleo 2, San Juan, Argentina
[f] Departamento de Zoonosis Provincial, Chacabuco 169, Catamarca, Argentina
[g] Cátedra de Parasitología y Enfermedades Parasitarias, UNR: Bv. Ovidio Lagos 1000, 2170 Casilda, Santa Fe, Argentina
[h] División Zoonosis Rurales, UNCPBA, Facultad Agronomía, Azul 7300, Argentina

ARTICLE INFO

Article history:
Received 19 October 2016
Accepted 7 February 2017
Available online 11 February 2017

Keywords:
Echinococcus granulosus sensu lato
South America
Genotypes
Neglected disease
Echinococcosis

ABSTRACT

The aim of this work was to determine *Echinococcus granulosus* sensu lato species and genotypes in intermediate and definitive hosts and in human isolates from endemic regions of Argentina and Brazil including those where no molecular data is available by a combination of classical and alternative molecular tools. A total of 227 samples were isolated from humans, natural intermediate and definitive hosts. Amplification of cytochrome *c* oxidase subunit I gene fragment was performed and a combination of *Alu*I digestion assay, High Resolution Melting analysis (HRM) assay and DNA sequencing was implemented for *Echinococcus* species/genotype determination. *E. granulosus* sensu stricto (G1) was found in sheep (n = 35), cattle (n = 67) and dogs (n = 5); *E. ortleppi* (G5) in humans (n = 3) and cattle (n = 108); *E. canadensis* (G6) in humans (n = 2) and *E. canadensis* (G7) in pigs (n = 7). We reported for the first time the presence of *E. ortleppi* (G5) and *E. canadensis* (G6) in humans from San Juan and Catamarca Argentinean provinces and *E. canadensis* (G7) in pigs from Cordoba Argentinean province. In this work, we widened molecular epidemiology studies of *E. granulosus* s. l. in South America by analyzing several isolates from definitive and intermediate hosts, including humans from endemic regions were such information was scarce or unavailable. The presence of different species/genotypes in the same region and host species reinforce the need of rapid and specific techniques for accurate determination of *Echinococcus* species such as the ones proposed in this work.

1. Introduction

Cystic echinococcosis (CE) is a chronic parasitic zoonosis caused by the larval stage of the cestode *Echinococcus granulosus* sensu lato (s. l.), which affects humans, domestic and wild mammals. *E. granulosus* s. l. requires two mammalian hosts to complete its life cycle: a definitive host (usually dogs or other canids) and an intermediate host (wild or livestock mammals) being humans intermediate accidental hosts. This neglected disease is endemic in Argentina, southern Brazil, Uruguay, Chile and mountainous regions of Peru and Bolivia. In South American countries, 29.556 human cases were reported between January 2009 to December 2014 with 2.9% lethality (820 deaths) and 3000 days of hospitalization [1]. *E. granulosus* s. l. is now considered as a complex composed of different species and genotypes. Several works have shown that some of them differ in features such as biochemical components [2], hooks morphology [3], fertile cyst development in natural [4] and experimental infections [5], intermediate host specificity, prepatent period [6], antigenicity [7], and rate of infection in humans (reviewed in [8,9]). Several molecular tools have been used for

* Corresponding author.
E-mail addresses: avilahg@hotmail.com (H.G. Avila), guilhermebrzos@gmail.com (G.B. Santos), marcecucher@gmail.com (M.A. Cucher), macchiaroli.natalia@gmail.com (N. Macchiaroli), perezmatiasgaston86@gmail.com (M.G. Pérez), germanbaldi@gmail.com (G. Baldi), jensenoscar52@gmail.com (O. Jensen), veroperez99@yahoo.com.ar (V. Pérez), raulopez_27@yahoo.com.ar (R. López), pnegro@fveter.unr.edu.ar (P. Negro), escialfa@yahoo.com.ar (E. Scialfa), zaha@cbiot.ufrgs.br (A. Zaha), henrique@cbiot.ufrgs.br (H.B. Ferreira), mrosenzvit@fmed.uba.ar (M. Rosenzvit), lkamenetzky@fmed.uba.ar (L. Kamenetzky).

http://dx.doi.org/10.1016/j.parint.2017.02.001
1383-5769/

polymorphism analysis in *Echinococcus* spp., such as restriction fragment length polymorphism (RFLP) [10], multiplex-PCR [11], High Resolution Melting analysis (HRM) [12–14], loop-mediated isothermal amplification (LAMP) [15–18], and PCR gene amplification followed by sequencing [19–22]. The gene loci most frequently employed are mitochondrial genes, such as cytochrome *c* oxidase subunit 1 (cox1) and nicotinamide dehydrogenase subunit 1 (ND1) genes. Recently, phylogenetic analyses over complete mitochondrial genomes [23,24] were implemented to define species in the *Echinococcus* genus. Currently, *E. granulosus* s. l. is considered to be composed by *Echinococcus granulosus* sensu stricto (s. s.) (G1, G2 and G3 genotypes), *E. equinus* (G4 genotype), *Echinococcus ortleppi* (G5 genotype), *Echinococcus canadensis* (G6, G7, G8, G10 genotypes) and *Echinococcus felidis* ('lion strain'). In Argentina, a total of six genotypes corresponding to three species are circulating in livestock: *Echinococcus granulosus* s. s. G1 in sheep, cattle, goat, and pig (49.3%); *E. granulosus* s. s. G2 in sheep and cattle (1.7%); *E. granulosus* s. s. G3 in sheep (0.3%); *E. ortleppi* G5 in cattle (2.6%); *E. canadensis* G6 in goats and cattle (8.4%); and *E. canadensis* G7 in pigs (37.7%). In humans, three species corresponding to four genotypes were reported, *E. granulosus* s. s. G1 and G2, *E. canadensis* G6 and *E. ortleppi* G5. *E. granulosus* s. s. G1 was the most prevalent (54.2%, 45/83), followed by *E. canadensis* (36.1%, 30/83). Until now, only two human cases of *E. ortleppi* (G5) were reported. Despite the high number of samples analyzed over the last 10 years in Argentina [8,20,25,26] there are still regions without parasite genetic information. The human population living in these regions is exposed to an increased risk of becoming infected by *Echinococcus* due to their low socio economical level. In Brazil, genetic analyses in natural intermediate hosts are only available for cattle and pig. From a total of 815 isolates from cattle reviewed in Cucher et al. [8], 58.2% were *E. granulosus* s. s. G1, 40.9% *E. ortleppi* G5 and only one isolate belongs to *E. canadensis* G7. In pigs, *E. canadensis* G7 was described in 3 isolates and *E. granulosus* s. s. G1 in two isolates. In spite of the fact that there are few human cystic echinococcosis cases with genotype information from Brazil (N = 6), two species were found, *E. granulosus* s. s. G1 and *E. ortleppi* G5 [27]. More isolates from different species and geographic origins are needed to certainly describe the epidemiological situation of echinococcosis in Brazil. Previously, members of our group have developed a rapid and effective molecular tool based on HRM that allowed discriminating among species and genotypes from *Echinococcus* genus [12]. In this work, we aimed at determining *E. granulosus* s. l. species and genotypes in intermediate, including humans, and definitive hosts from endemic regions of Argentina and southern Brazil including those where no molecular data are available and to evaluate the usefulness of HRM technique in combination with other tools to perform epidemiological studies.

2. Materials and methods

2.1. Parasite material

A total of 227 samples were isolated and genomic DNA was obtained from fresh or 70% ethanol preserved isolates from humans and naturally infected animals from Argentina and southern Brazil. Material from hydatid cysts was observed under a light microscopy to verify presence of protoscoleces. In this study, an *E. granulosus* s. l. isolate from an intermediate host, refers to the protoscoleces or germinal layer obtained from a single hydatid cyst. In the definitive host, an isolate refers to a single adult. The host and geographical origin of the isolates analyzed in this study are shown in Table 1 and Supplementary material Table S1.

2.2. DNA extraction

DNA isolation from protoscoleces was performed as follows: protoscoleces were washed three times in PBS 1× and then lysed with 100 μl of 100 mM Tris–HCl pH 8, 250 mM NaCl, 0.5% SDS, 100 mM EDTA and 100 μg/ml proteinase K at 56 °C for 2 h. Then, DNA was extracted using the phenol–chloroform method [28], resuspended in nuclease-free water and stored at −20 °C. DNA isolation of samples from adult worms or hydatid cysts without protoscoleces was made by DNeasy Blood & Tissue Kit® (QIAGEN) following the manufacturer's instructions. DNA concentration was determined using a Nanodrop 2000 and DNA integrity was assessed by electrophoresis in 1.5% agarose gel stained with GelRed® (Biotium). DNA prepared from all analyzed samples was undegraded.

Table 1
Species/genotype, host, geographic origin, number of isolates, from the 227 total *Echinococcus* samples analyzed.

Echinococcus species/genotype	Host	Geographic origin (country/state or province)	Number of isolates	Total by host
E. granulosus sensu stricto	Sheep	Argentina/Chubut	35	35
	Cattle	Argentina/Buenos Aires	2	67
		Argentina/Chubut	3	
		Argentina/Santa Fe	1	
		Brasil/Rio Grande do Sul	61	
	Dog	Argentina/Buenos Aires	2	5
		Argentina/Chubut	3	
E. ortleppi (G5)	Human	Argentina/Catamarca	2	3
		Argentina/San Juan	1	
	Cattle	Brasil/Rio Grande do Sul	108	108
E. canadensis (G6)	Human	Argentina/Catamarca	1	2
		Argentina/San Juan	1	
E. canadensis (G7)	Pig	Argentina/Buenos Aires	3	7
		Argentina/Cordoba	4	
Total				227

2.3. Genotyping

2.3.1. Amplification of cytochrome c oxidase subunit I (cox1) gene fragment

Amplification of the mitochondrial cox1 gene was made by PCR based on [19] but with minor modifications as described in [5]. Briefly, a 441 bp cox 1 gene fragment was amplified using the primers described in (5): 5′-TTTGGGCATCCTGAGGTT-3′ (forward) and 5′-TAAAGAAAGAACATAATGAAAATG-3′ (reverse) in the following reaction mixture: 30 ng of DNA template, 5 mM dNTP, 5 pmol of each primer, 1.2 mM $MgCl_2$, 1 U Taq DNA polymerase (Invitrogen, USA), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 μM of SYTO 13 (Life technologies®) in a total volume of 20 μl. The PCRs were performed in a 7500 thermal cycler (Applied Biosystems®) under primer annealing touchdown strategy, starting with 55 °C and decreasing 1 °C every 2 cycles during the first 20 cycles, followed by 15 more cycles at 45 °C, always for 30 s. The first denaturing step (95 °C) lasted 5 min, and 30 s in the remaining cycles. Extension was performed at 72 °C for 60 s in the first 34 cycles and for 5 min in the last cycle.

2.3.2. AluI digestion assay

A total of 5 μl of cox1 PCR product was digested with 4 U of *Alu*I (Invitrogen), 1× Buffer Tango in a final volume of 10 μl. The samples were incubated at 37 °C during 3.5 h; the final inactivation step was performed at 65 °C during 30 min. The enzyme digestion products were visualized by electrophoresis in 3% agarose gel stained with GelRed® (Biotium).

2.3.3. High resolution melting analysis

The cox1 PCR product obtained was subjected to the HRM technique using the 7500 Fast (Applied Biosystems®). In this assay melting was conducted by increasing the temperature from 60 °C to 99 °C at ramping increments from 0.05 °C/s. The HRM analysis was carried out using the HRM software from Applied Biosystem (version 3.0.1) with normalization regions between 79.1–79.4 °C and 87.1–87.4 °C. A difference melting curve was produced with *E. canadensis* (G7) as baseline.

2.3.4. Sequencing

The *E. granulosus* s. l. genotype/species determination was performed by sequencing of the mitochondrial cox1 fragment. Sequencing by Sanger method was performed at Macrogen (Macrogen, South Korea). The sequences were aligned with reference genotype sequences: M84661 (G1), M84662 (G2), M84663 (G3), M84664 (G4), M84665 (G5), M84666 (G6), M84667 (G7), AB235848 (G8) and AF525457 (G10) using Clustal X software and manually edited by BioEdit software. Genotype/species determination was performed by the method of Maximum Likelihood using MEGA6 software. Distance matrices were constructed and the obtained trees were evaluated by the bootstrap on 500 replicates. The values of the nodes correspond to data obtained in over 50% of the replicates.

2.4. Echinococcus species/genotype and livestock density

The relationship between the abundance of each *Echinococcus* species/genotype and livestock density was analyzed by means of a Spearman correlation test, and these values were visualized as a star diagram [29]. Livestock density data was collected at a second order subnational unit (Department or Municipality) from the *Dirección de Análisis Económico Pecuario, Dirección Nacional de Estudios y Análisis Económicos*

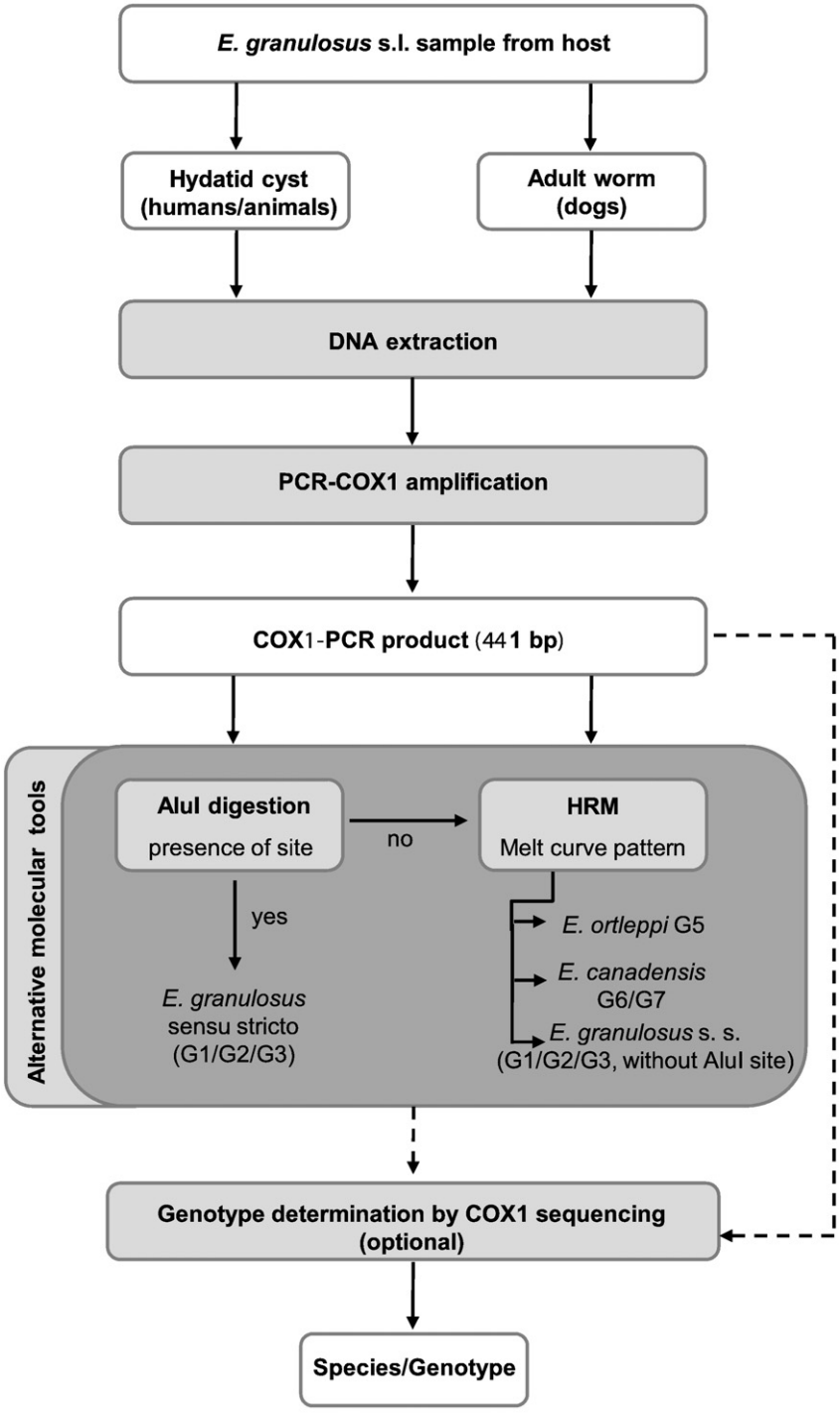


Fig. 1. Flowchart for *Echinococcus* species/genotype determination.

del Sector Pecuario (2016) Existencias Ganaderas. Buenos Aires, Argentina: Dirección Nacional de Sanidad Animal (SENASA) and *Instituto Brasileiro de Geografia e Estatística (IBGE) (2013) Pesquisa Pecuária Municipal 2013.*

3. Results

A total of 227 samples from human, cattle, pig, sheep and dogs were analyzed. We employed a combination of three methods over cox1 gene sequence for molecular determination of *Echinococcus* species (Fig. 1). *E. granulosus* s. s. (G1) was found in sheep (n = 35), cattle (n = 67) and dog (n = 5); *E. ortleppi* (G5) in humans (n = 3) and cattle (n = 108); *E. canadensis* (G6) in humans (n = 2) and *E. canadensis* (G7) in pigs (n = 7) (Table 1).

The *Alu*I digestion assay can differentiate *E. granulosus* s. s. from the other *Echinococcus* species due to the presence of the *Alu*I site in the majority of *E. granulosus* s. s. (G1–G3) cox1 sequences reported so far. The DNA fragments obtained after cox1-*Alu*I digestion are two products of 208 bp and 233 bp, respectively (Fig. 2A). A total of 219 samples were analyzed by *Alu*I digestion and 99 samples (45.2%) of them showed two bands indicating the presence of *E. granulosus* s. s. (G1–G3) and allowed us to assume that 54.8% (120 samples) of the samples analyzed did not belong to *E. granulosus* s. s. species. Then, with HRM assay three melting curves were obtained, each belonging to *E. granulosus* s. s., *E. ortleppi*, and *E. canadensis* species (Fig. 2B), allowing to identification at species level. A total of 215 samples were successfully identified with this method (Supplementary material Table S1) showing the potential of HRM technique for epidemiological studies with high number of samples. Finally, all human samples and a subset of isolates from animals, totaling 36 samples, were confirmed by cox1 sequencing. The results showed that 66.7% (24/36) of the analyzed isolates belonged to *E. granulosus* s. s. (G1), 8.3% (3/36) to *E. ortleppi* (G5), 5.6% (2/36) to *E. canadensis* (G6) and 19.4% (7/36) to *E. canadensis* (G7) (Fig. 2C) accordingly with *Alu*I digestion and HRM assays (Supplementary material Fig. S1). Alternative techniques, *Alu*I digestion and HRM assays, used in this work were useful for the identification of species/genotype in less than 48 h. The geographic and host origin of the samples were also analyzed. Interestingly, the samples obtained are representative of a wide area from Argentina and Southern Brazil (Fig. 3). In this work we reported for the first time the presence of *E. ortleppi* (G5) and *E. canadensis* (G6) in human cases from San Juan and Catamarca provinces. Also, it is the first time that the presence of *E. canadensis* (G7) in pigs is reported in the province of Cordoba.

The correlation analysis between species/genotypes of *E. granulosus* s. l. and livestock density (Fig. 4 and Supplementary material Table S2) indicates that *E. granulosus* s. s. (G1) is present in areas with high sheep density (correlation coefficient = 0.68), supporting the main role of this livestock species as intermediate host [4,30]. On the other hand, the regions where we found *E. ortleppi* (G5) have high density of cattle and sheep (correlation coefficient = 0.45 and 0.33, respectively), both previously described as frequent hosts of this parasite species [8]. *E. canadensis* (G6), found in humans, coincides in its distribution with goats and American camelids livestock (though correlation coefficients were not significant perhaps due to the low number of samples from this species in our data set). Finally, *E. canadensis* (G7) was found in areas with high density of pigs (correlation coefficient = 0.46) as was already described [8,31], but also in areas of high density of American camelids encouraging new studies about the role of south American camelids as intermediate host of this species/genotype.

4. Discussion

E. granulosus s. l. samples from intermediate (including humans) and definitive hosts and areas were identified in this work. The analyzed samples are representative of a wide variability in areas of Argentina and southern Brazil. Here we report for the first time the presence of humans infected with *E. ortleppi* (G5) and *E. canadensis* (G6) in the province of San Juan where no previous molecular epidemiology data were published. It is also the first report of *E. ortleppi* (G5) infected humans in the province of Catamarca. These results suggest the need to extend the repertoire of genotyped hydatid cysts from slaughterhouses (intermediate hosts) and adult worms from arecoline purges/collected faeces (definitive hosts) in order to determine if the life cycles of these species are present in San Juan and Catamarca. These types of studies should be extended to all provinces where the circulating genotypes are not known, or data are scarce, as in the province of Cordoba where the presence of *E. canadensis* (G7) in pigs was previously unknown. The results obtained from the other analyzed provinces (Santa Fe, Buenos Aires, Catamarca and Chubut) are consistent with the previously reported [8,20,25]. Also for isolates collected from Brazil, the species/genotypes here obtained were consistent with those described by [32].

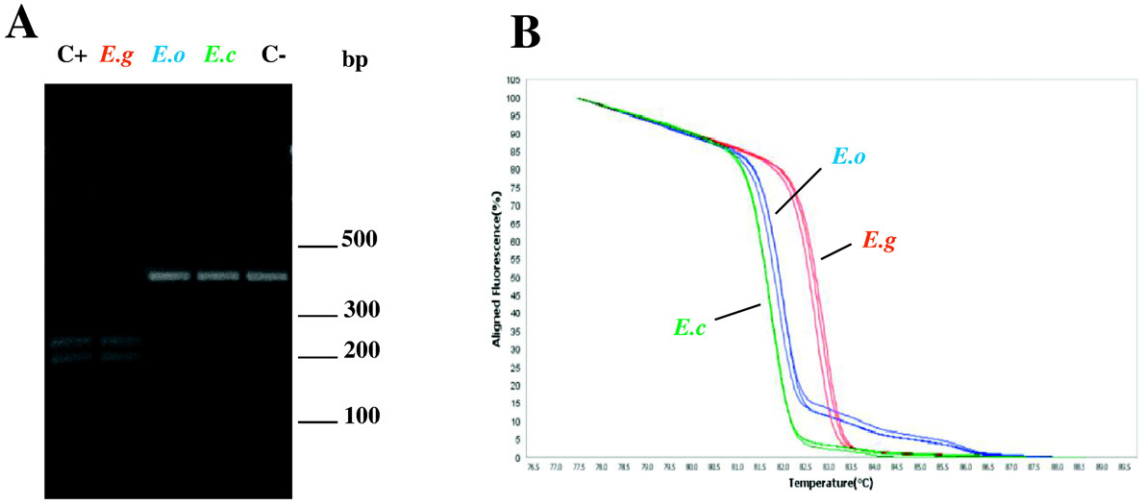
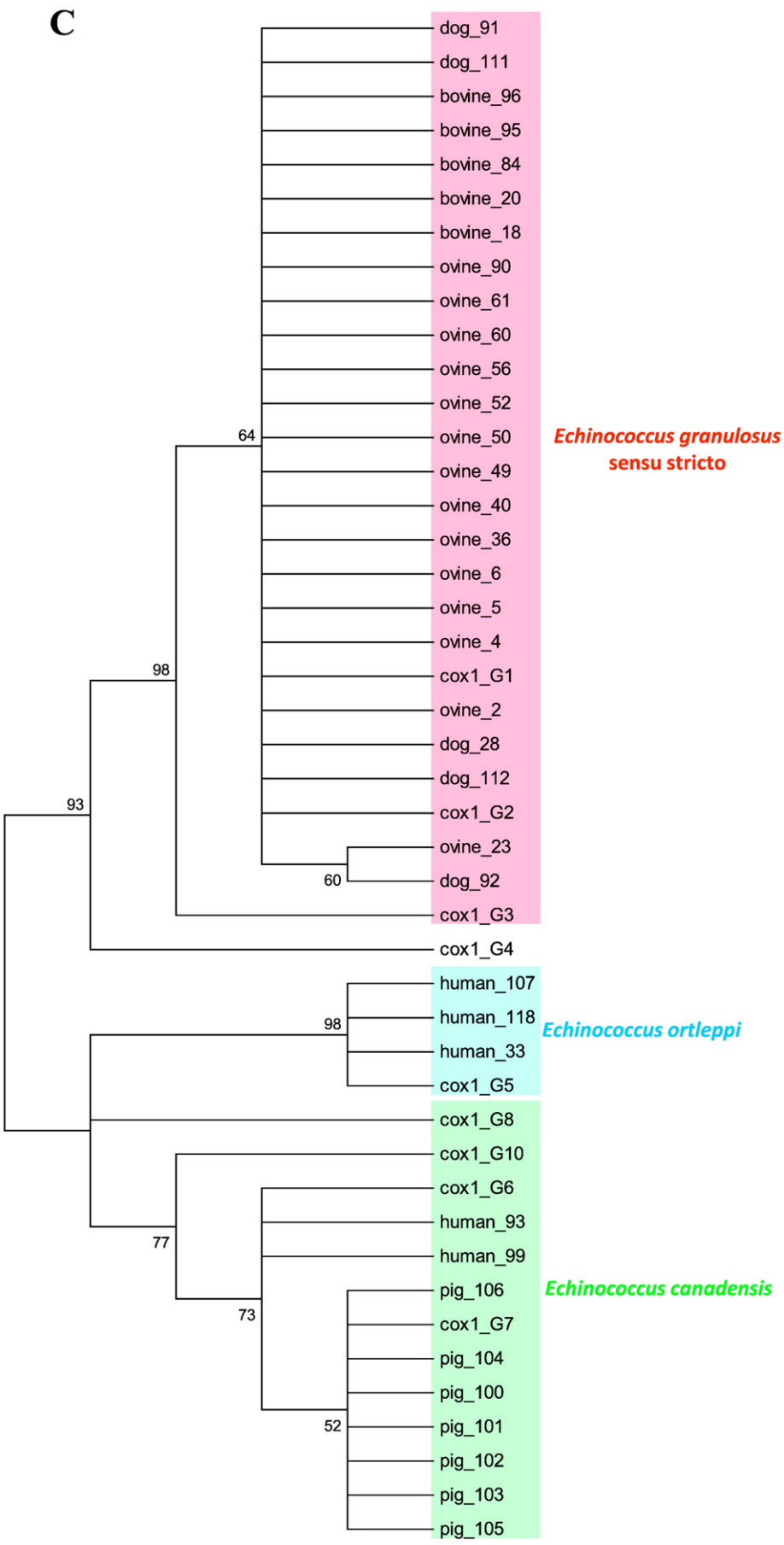


Fig. 2. Molecular determination of *Echinococcus* species. Representative samples of each *Echinococcus* species were analyzed by three methods. (A) Gel electrophoresis of DNA fragments from *Alu*I digestion assay, Eg: *E. granulosus* s. s. G1, Eo: *E. ortleppi* G5, Ec: *E. canadensis* G6–G7, C+: positive control, C−: negative control; (B) High Resolution Melting (HRM), in red, *E. granulosus* s. s. G1, in blue *E. ortleppi* G5, and in green curves belong to samples from *E. canadensis* G7. (C) Maximum likelihood tree of representative isolates and reference sequences of *Echinococcus* species and genotypes: M84661 (G1), M84662 (G2), M84663 (G3), M84664 (G4), M84665 (G5), M84666 (G6), M84667 (G7), AB235848 (G8) and AF525457 (G10). In red *E. granulosus* s. s. G1, G2 and G3, in blue *E. ortleppi* G5, and in green *E. canadensis* G6–G10. Bootstrap values above 50% are shown at nodes.



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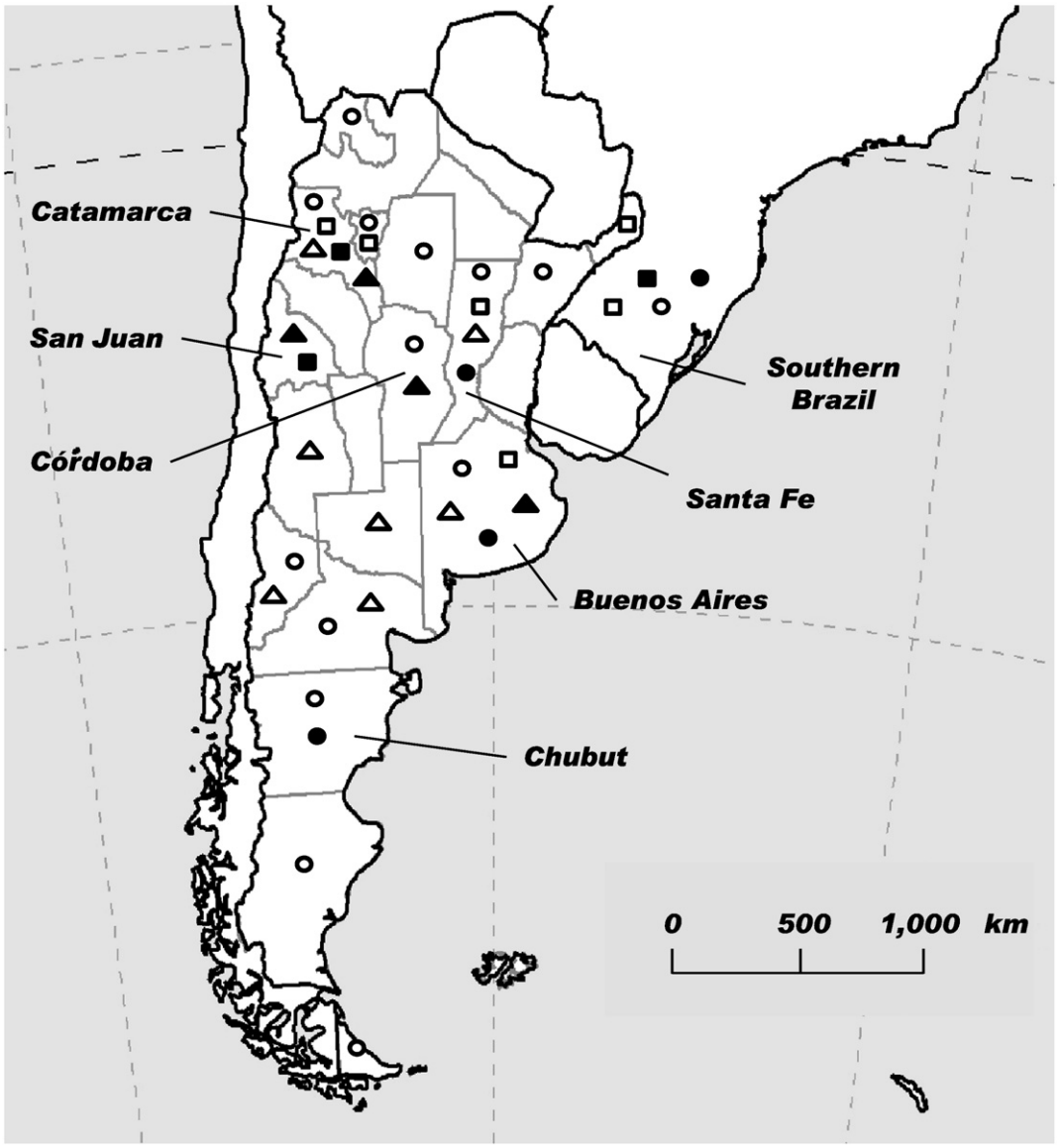
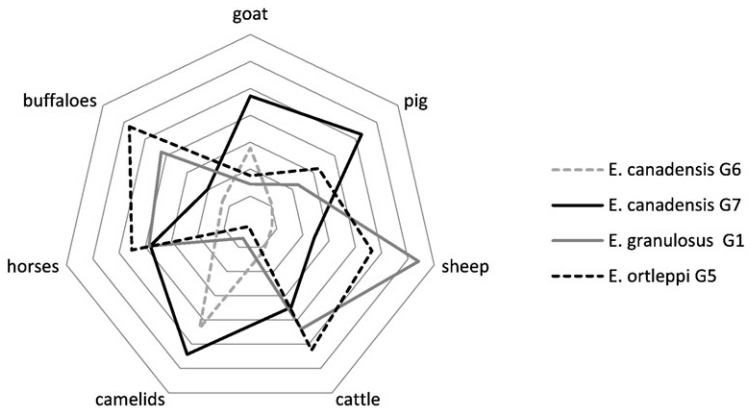


Fig. 3. *Echinococcus granulosus* sensu lato species from Argentina and Brazil. Geographical origin of the samples and species/genotype identified in this work. Circles: *E. granulosus* s. s. G1, G2 and G3, Squares: *E. ortleppi* G5, Triangles: *E. canadensis* G6 and G7. Solid: this work, Open: previous work [8]. Argentinian provinces and Southern Brazil region analyzed in this work are indicated by name.

In this work the performance of HRM and *Alu*I digestion assay, as an alternative tool to sequencing was also evaluated. The results obtained showed that it was a rapid and effective technique allowing the identification of *E. granulosus* s. s. (G1-G3), *E. ortleppi* (G5), and *E. canadensis* (G6-G7) in a high number of samples (more than 200) from different localities. Furthermore, the *Alu*I digestion assay allows rapid identification of the species *E. granulosus* s. s. with low DNA concentration allowing analyzing samples with few parasite material available. While this technique enables rapid and simple identification of *E. granulosus* s. s., it fails to distinguish species when the *Alu*I site is not present in the cox1 sequence, such as in *E. canadensis*, *E. ortleppi* and a few *E. granulosus* s. s. haplotypes with a mutation in the *Alu*I site. For this reason, here we propose that it can be used as a quick screening, followed by sequencing or HRM curve analysis in case it is negative (sequences without *Alu*I site). From these findings, a protocol of molecular identification of species/genotypes including alternative techniques used in this work was proposed (Fig. 1). An important point is that the alternative tools developed/evaluated in this work allowed the identification of *Echinococcus* samples in less than 48 h.

In contrast with previous reports, human cases analyzed in this work did not show the main species infecting humans worldwide, the *E. granulosus* s. s. (G1), but showed *E. canadensis* (G6) and *E. ortleppi* (G5) as causative agents of echinococcosis (Table 1). It is alarming that all human cases were from young people (7, 9, 12, 20 and 29 years old) indicating that this parasite is actively infecting humans in Argentina. Particularly, *E. ortleppi* (G5) is considered poorly infective to humans since only nine humans cases were reported so far [8]. The discovery of three new human cases of this species, unfortunately children, suggests that it is an emerging species in South America. In the province of Catamarca cases of dogs with *E. ortleppi* (G5) and *E. canadensis* (G6) and humans with *E. canadensis* (G6) were reported [20]. It is known that goats are frequent intermediate hosts of *E. canadensis* (G6) [8], however, they also could act as reservoirs of *E. ortleppi* (G5) since 3 goats from Kenya have been described to have fertile cysts from this parasite species [33]. It would be interesting to evaluate goats from Catamarca province to elucidate the epidemiological situation in mountain regions of this province with high goat density. Also, a similar epidemiological situation could be found in San Juan



species	goat	pig	sheep	cattle	camelids	horses	buffaloes
E. canadensis/G6	-0.04	-0.39	-0.44	-0.37	0.27	-0.35	-0.34
E. canadensis/G7	0.34	0.46	-0.11	0.10	0.48	0.16	-0.20
E. granulosus s.s/G1	-0.31	-0.15	0.68	0.27	-0.47	0.17	0.25
E. ortleppi/G5	-0.25	0.05	0.33	0.45	-0.57	0.30	0.55

Fig. 4. Correlation between abundance of each *Echinococcus* species/genotype and livestock density. The length of each radius of the star diagram represents the values of the variables in each observation (Spearman correlation coefficient).

province, where humans can be infected with *E. ortleppi* (G5) and *E. canadensis* (G6) and goats are de most prevalent livestock species. *E. canadensis* (G6), found in humans, coincides in its distribution with goats and American camelids livestock. Thus, it would be interesting to analyze more humans and livestock from the same region to evaluate if these host species would be the natural reservoir of *E. canadensis* (G6), as we previously suggested [20]. Also, it is essential to increase the number of analyses of samples from South American camelids, to conclude whether they act as a reservoir of *E. granulosus* s. s (G1), as already described in 4 alpacas in Peru [34]; or of some other species such as *E. canadensis* (G6–G7), as it was reported for Middle East camelids reviewed in [8]. Samples analyzed from Brazil confirmed the results obtained by Balbinotti et al. [32] which indicates that cattle harbors both *E. ortleppi* (G5) and *E. granulosus* s. s. (G1) species. Unfortunately, no human cases were plausible to sample from this country but considering the presence of *E. ortleppi* (G5) in humans from Argentina, cattle should be considered as an active reservoir of *Echinococcus* and more control efforts have to be implemented in this type of livestock. Also, more livestock species from San Juan and Catamarca provinces have to be analyzed to determine all possible natural reservoirs of *E. ortleppi* (G5).

It is known that intraspecific variation present in the genotypes of *E. granulosus* s. l. can affect life cycle patterns such as host specificity, speed of development, pathogenicity and antigenicity. This may have important consequences in the design and development of vaccines, diagnostic and therapy strategies and the implementation of echinococcosis control programs. In addition, it is important to broaden epidemiological and molecular knowledge of each area. All kind of advances in understanding the molecular epidemiology, considering the genetic variability that presents each species, can help the control programs to implement robust tools against the species that are affecting each geographic region. In this work, we showed the possibility of performing such molecular epidemiology studies by combining classical and alternative techniques that provided fundamental information to be taken into account in control programs of cystic echinococcosis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2017.02.001.

Acknowledgements

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, projects PICT 2013 N° 2121, PICT-CABBIO 2012 N° 3044, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) project PIP 114 2011, CAPG-BA 070/13, CAPES-MinCyT BR 1204 2013, MinCyT-CAPES BR/RED 1413 2015 and Fundación Mundo Sano 2016.

References

[1] PANAFTOSA - PAHO/WHO, Equinococosis, Vet. Public Heal 1 (2015).
[2] D.P. McManus, C. Bryant, Biochemistry, physiology and molecular biology of *Echinococcus*, in: R.C.A. Thompson, A.J. Lymbery (Eds.), Echinococcus Hydatid Dis, CAB International, Oxfordshire, UK, 1995.
[3] S.V. Soriano, N.B. Pierangeli, L.A. Pianciola, M. Mazzeo, L.E. Lazzarini, M.F. Debiaggi, H.F.J. Bergagna, J.A. Basualdo, The optimum cut-off value to differentiate *Echinococcus granulosus* sensu stricto from other species of *E. granulosus* sensu lato using larval rostellar hook morphometry, J. Helminthol. (2013) 1–8, http://dx.doi.org/10.1017/S0022149X13000473.
[4] R.C.A. Thompson, The taxonomy, phylogeny and transmission of *Echinococcus*, Exp. Parasitol. 119 (2008) 439–446, http://dx.doi.org/10.1016/j.exppara.2008.04.016.
[5] M. Cucher, G. Mourglia-Ettlin, L. Prada, H. Costa, L. Kamenetzky, C. Poncini, S. Dematteis, M.C. Rosenzvit, *Echinococcus granulosus* pig strain (G7 genotype) protoscoleces did not develop secondary hydatid cysts in mice, Vet. Parasitol. 193 (2013) 185–192.
[6] R.C.A. Thompson, Growth, segmentation and maturation of the British horse and sheep strains of *Echinococcus granulosus* in dogs, Int. J. Parasitol. 7 (1977) 281–285.
[7] C.A. Alvarez Rojas, C.G. Gauci, M.W. Lightowlers, Antigenic differences between the EG95-related proteins from *Echinococcus granulosus* G1 and G6 genotypes: implications for vaccination, Parasite Immunol. 35 (2013) 99–102.
[8] M.A. Cucher, N. Macchiaroli, G. Baldi, F. Camicia, L. Prada, L. Maldonado, H.G. Avila, A. Fox, A. Gutiérrez, P. Negro, R. López, O. Jensen, M. Rosenzvit, L. Kamenetzky, Cystic echinococcosis in South America: systematic review of species and genotypes of *Echinococcus granulosus* sensu lato in humans and natural domestic hosts, Trop. Med. Int. Heal. 21 (2016) 166–175, http://dx.doi.org/10.1111/tmi.12647.

[9] C.A. Alvarez Rojas, T. Romig, M.W. Lightowlers, *Echinococcus granulosus* sensu lato genotypes infecting humans—review of current knowledge, Int. J. Parasitol. 44 (2014) 9–18, http://dx.doi.org/10.1016/j.ijpara.2013.08.008.
[10] J. Bowles, D.P. McManus, Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method, Mol. Biochem. Parasitol. 57 (1993) 231–239, http://dx.doi.org/10.1016/0166-6851(93)90199-8.
[11] G. Boubaker, N. Macchiaroli, L. Prada, M.A. Cucher, M.C. Rosenzvit, I. Ziadinov, P. Deplazes, U. Saarma, H. Babba, B. Gottstein, M. Spiliotis, A multiplex PCR for the simultaneous detection and genotyping of the *Echinococcus granulosus* complex, PLoS Negl. Trop. Dis. 7 (2013), e2017. http://dx.doi.org/10.1371/journal.pntd.0002017.
[12] G.B. Santos, S.M. Espínola, H.B. Ferreira, R. Margis, A. Zaha, Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach, Parasites & Vectors. 6 (2013) 327, http://dx.doi.org/10.1186/1756-3305-6-327.
[13] S. Rostami, S. Talebi, Z. Babaei, M. Sharbatkhori, N. Ziaali, H. Rostami, M.F. Harandi, High resolution melting technique for molecular epidemiological studies of cystic echinococcosis: differentiating G1, G3, and G6 genotypes of *Echinococcus granulosus* sensu lato, Parasitol. Res. 112 (2013) 3441–3447, http://dx.doi.org/10.1007/s00436-013-3523-z.
[14] A.H. Safa, M.F. Harandi, M. Tajaddini, M. Rostami-Nejad, M. Mohtashami-Pour, N. Pestehchian, Rapid identification of *Echinococcus granulosus* and *E. canadensis* using high-resolution melting (HRM) analysis by focusing on single nucleotide polymorphism, Jpn. J. Infect. Dis. (2015), http://dx.doi.org/10.7883/yoken.JJID.2015.161.
[15] M. Wassermann, U. Mackenstedt, T. Romig, A loop-mediated isothermal amplification (LAMP) method for the identification of species within the *Echinococcus granulosus* complex, Vet. Parasitol. 200 (2014) 97–103, http://dx.doi.org/10.1016/j.vetpar.2013.12.012.
[16] X.W. Ni, D.P. McManus, Z.Z. Lou, J.F. Yang, H. Bin Yan, L. Li, H.M. Li, Q.Y. Liu, C.H. Li, W.G. Shi, Y.L. Fan, X. Liu, J.Z. Cai, M.T. Lei, B.Q. Fu, Y.R. Yang, W.Z. Jia, A comparison of loop-mediated isothermal amplification (LAMP) with other surveillance tools for *Echinococcus granulosus* diagnosis in canine definitive hosts, PLoS One 9 (2014), http://dx.doi.org/10.1371/journal.pone.0100877.
[17] H. Salant, I. Abbasi, J. Hamburger, The development of a loop-mediated isothermal amplification method (LAMP) for *Echinococcus granulosis* coprodetection, Am.J.Trop. Med. Hyg. 87 (2012) 883–887, http://dx.doi.org/10.4269/ajtmh.2012.12-0184.
[18] M.E. Ahmed, M.H. Eldigail, F.M. Elamin, I.A. Ali, M.P. Grobusch, I.E. Aradaib, Development and evaluation of real-time loop-mediated isothermal amplification assay for rapid detection of cystic echinococcosis, BMC Vet. Res. 1–10 (2016), http://dx.doi.org/10.1186/s12917-016-0809-2.
[19] J. Bowles, D. Blair, D.P. McManus, Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing, Mol. Biochem. Parasitol. 54 (1992) 165–174.
[20] L. Kamenetzky, A.M. Gutierrez, S.G. Canova, K.L. Haag, E.A. Guarnera, A. Parra, G.E. García, M.C. Rosenzvit, G.E. Garc, M.C. Rosenzvit, Several strains of Echinococcus granulosus infect livestock and humans in Argentina, Infect. Genet. Evol. 2 (2002) 129–136 http://www.ncbi.nlm.nih.gov/pubmed/12797989.
[21] J.-M. Bart, K. Bardonnet, M.C.B. Elfegoun, H. Dumon, L. Dia, D.A. Vuitton, R. Piarroux, J.M. Bart, K. Bardonnet, M.C.B. Elfegoun, H. Dumon, L. Dia, D.A. Vuitton, R. Piarroux, *Echinococcus granulosus* strain typing in North Africa: comparison of eight nuclear and mitochondrial DNA fragments, Parasitology. 128 (2004) 229–234, http://dx.doi.org/10.1017/S0031182003004359.
[22] P.L. Moro, M. Nakao, A. Ito, P.M. Schantz, C. Cavero, L. Cabrera, Molecular identification of *Echinococcus* isolates from Peru, Parasitol. Int. 58 (2009) 184–186, http://dx.doi.org/10.1016/j.parint.2009.01.005.
[23] M. Nakao, D.P. McManus, P.M. Schantz, P.S. Craig, A. Ito, A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes, Parasitology 134 (2007) 713–722, http://dx.doi.org/10.1017/S0031182006001934.
[24] M. Nakao, A. Lavikainen, T. Yanagida, A. Ito, Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae), Int. J. Parasitol. 43 (2013) 1017–1029, http://dx.doi.org/10.1016/j.ijpara.2013.06.002.
[25] M. Rosenzvit, L.-H. Zhang, L. Kamenetzky, S.G. Canova, E.A. Guarnera, D.P. McManus, Genetic variation and epidemiology of *Echinococcus granulosus* in Argentina, Parasitology 118 (1999) 523–530.
[26] L. Kamenetzky, S.G. Canova, E.A. Guarnera, M.C. Rosenzvit, *Echinococcus granulosus*: DNA extraction from germinal layers allows strain determination in fertile and nonfertile hydatid cysts, Exp. Parasitol. 95 (2000) 122–127, http://dx.doi.org/10.1006/expr.2000.4518.
[27] M.L. de la Rue, K. Takano, J.F. Brochado, C.V. Costa, A.G. Soares, K. Yamano, K. Yagi, Y. Katoh, K. Takahashi, Infection of humans and animals with Echinococcus granulosus (G1 and G3 strains) and E. ortleppi in Southern Brazil, Vet. Parasitol. 177 (2011) 97–103, http://dx.doi.org/10.1016/j.vetpar.2010.11.018.
[28] J. Sambrook, D.W. Russell, Molecular Cloning - Sambrook & Russel, 2001, http://dx.doi.org/10.1002/humu.1186.abs.
[29] J.M. Chambers, W.S. Cleveland, B. Kleiner, P.A. Tukey, Graphical Methods for Data Analysis, 1983.
[30] D.P. McManus, Current status of the genetics and molecular taxonomy of *Echinococcus* species, Parasitology 140 (2013) 1617–1623, http://dx.doi.org/10.1017/S0031182013000802.
[31] J.C. Scott, J. Stefaniak, Z.S. Pawlowski, D.P. McMANUS, Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*, Parasitology 114 (1997) 37–43, http://dx.doi.org/10.1017/S0031182096008062.
[32] H. Balbinotti, G.B. Santos, J. Badaraco, A.C. Arend, D.A. Graichen, K.L. Haag, A. Zaha, *Echinococcus ortleppi* (G5) and *Echinococcus granulosus* sensu stricto (G1) loads in cattle from Southern Brazil, Vet. Parasitol. 188 (2012) 255–260, http://dx.doi.org/10.1016/j.vetpar.2012.04.007.
[33] H. Mbaya, J. Magambo, S. Njenga, E. Zeyhle, C. Mbae, E. Mulinge, M. Wassermann, P. Kern, T. Romig, *Echinococcus* spp. in central Kenya: a different story, Parasitol. Res. 113 (2014) 3789–3794, http://dx.doi.org/10.1007/s00436-014-4045-z.
[34] E. Sánchez, O. Cáceres, C. Náquira, E. Miranda, F. Samudio, O. Fernandes, *Echinococcus granulosus* genotypes circulating in alpacas (*Lama pacos*) and pigs (*Sus scrofa*) from an endemic region in Peru, Mem. Inst. Oswaldo Cruz 107 (2012) 275–278, http://dx.doi.org/10.1590/S0074-02762012000200019.