High intraspecific variability of *Echinococcus granulosus sensu stricto* in Chile

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Abstract

Echinococcus granulosus sensu stricto is the major cause of cystic echinococcosis in most human and animal cases in the world and the most widespread species within the *E. granulosus sensu lato* complex. *E. granulosus s.s*. remains endemic in South America together with other species of the *Echinococcus* genus, especially in some areas in Argentina, Brazil, Chile and Peru. Except for a single human case caused by *E. canadensis* (G6) described in the literature, only *E. granulosus s.s*. has been found in the Chilean territory. In the current study 1,609bp of the *cox1* gene from 69 Chilean isolates of *E. granulosus* s.s. from humans and animals were analysed. In total, 26 *cox1* haplotypes were found, including the widespread haplotype EG01 (22 isolates) and also EGp1 (5), EgRUS7 (1), EgAus02 (1) and EgAus03 (2). Twenty-one different haplotype not previously described were identified from 38 Chilean isolates designated EgCL1-EgCL21. Previous work had described low variability of *E. granulosus s.s*. in South America, based on isolates from Peru. Results obtained in this work challenge the previously described idea of the low diversity of the parasite in South America, and warrant future investigation on the origin and spread of the parasite in the continent after the Spanish arrival.

Cystic echinococcosis (CE) is endemic in Chile as well as in several other South American countries including Argentina, Peru, Uruguay and Brazil [1]. The parasite is able to survive the variety of weather conditions present in the Chilean territory ranging from the arid climate in the north to subpolar oceanic in the extreme south. Within the sixteen administrative regions into which the country is divided (numbered from I to XV plus the capital Santiago), CE is highly endemic in Coquimbo (IV), La Araucania (IX), Aysen (XI) and Magallanes (XII) [2, 3]. CE is the second most important cause of condemnation of viscera in livestock (following infection with *Fasciola hepatica*) [4]. Including the cost of animal losses, estimates of the economic burden of CE in Chile are up to USD 14.35 million/year [3]. Only two studies have investigated the molecular variability of *E*. *granulosus* in Chile. Manterola *et al* [5], studied 20 human samples, confirming the presence of *E*. *granulosus s.s.* in 19 isolates and *E*. *canadensis* (G6 genotype) in a single case following the sequencing of a 366bp section of the *cox1* gene and a 471bp section of the *nad1* gene. Subsequently, using a similar methodology, Espinoza *et al* [6] analysed 15 hydatid cysts from cattle and humans, finding that they were all *E*. *granulosus s*.*s*.

There is growing evidence that using longer sequences of *cox1* or other genes allows the description of intraspecific variability of *Echinococcus spp*. at a higher resolution than using the one established with the G-definition [7-9]. In fact, Yanagida *et al* [8] have suggested that due to the lack of clarity in how some samples have been defined as G1, G2 and G3 in databases, the validity of these genotypes of *E*. *granulosus s*.*s*. is now needed to be verified by analysing longer sequences of mtDNA and/or nuclear genes from the sources that these genotypes were originally described. Moreover, Romig *et al* [10] reviewed the available data in GenBank and found that using the original G-definition of *cox1* (366bp sequences), a large proportion of the 137 haplotypes, based on the 1,609bp of the *cox1* gene, are not

homologous with either G1, G2 or G3. In South America, a single study has undertaken an analysis of haplotypes of *E*. *granulosus s.s*. using 1,609bp of the *cox1* gene. This research described only three haplotypes of *E*. *granulosus s.s*. in 30 samples isolated from livestock in Peru suggesting low variability of this parasite in South America [8]. In the present study, we challenge this idea, as an initial step of the analysis of the microdiversity of *E*. *granulosus s.s*. in South America, we sought to analyse isolates of *E*. *granulosus s.s*. from different regions in Chile using a sequence of 1,609bp of the *cox1* gene.

Protoscoleces or germinal membranes were extracted from individual hydatid cysts obtained from livestock animals at abattoirs located in different parts of Chile. Additionally, thirteen human CE samples from Chilean patients from the Hospital Hernan Henriquez Aravena in Temuco, Araucanía (IX) were used in this study. Partial *cox1* gene sequences (366bp) for the human samples had been published by Manterola *et al* [5]. Seven faecal samples from naturally infected dogs were obtained from an endemic area in the Coquimbo region. Supplementary Table 1 shows detailed information on the number, origin and hosts from which the 69 isolates used in the current study were collected. An isolate was defined as material derived from a single cyst (protoscoleces or germinal membrane) and an individual faecal sample in the case of isolates from dogs. DNA was extracted using phenol:chloroform:isoamylic alcohol as previously described [11]. For the faecal samples from dog, DNA was isolated using the ISOLATE Faecal DNA Kit (Bioline) following manufacturer instructions. Amplification of the *cox1* gene and Sanger sequencing was performed in two steps; also the analysis of electropherograms, identification of haplotypes, networks analyses, diversity and neutrality indices were computed as previously described [7].

The sequence of the c*ox1* gene (1,609bp) was obtained from all the 69 isolates which were all identified as *E*. *granulosus s.s.* revealing a high degree of genetic variability. Twenty two isolates were identified as the EG01 haplotype (accession number: JQ250806). Five isolates were assigned to the known haplotypes EGp1 (AB522646), two as EgAus03 (KT968704), one as EgAus02 (KT968703) and one as EgRUS7 (AB777904). Thirty-eight other samples were assigned to twenty-one haplotypes not previously described, and designated EgCL1-EgCL21 (accession numbers KX227116-KX227136 respectively) (Figure 1 A). Supplementary **Table 2** shows the position in which each haplotype differs in comparison with the reference haplotype EG01, in 15 positions nucleotide changes lead to non-synonymous amino acid substitutions. Human isolates used in this study were infected by 8 different haplotypes: three samples were identified as the common haplotype EG01, one as EGp1, 4 isolates shared the same sequence and were designated as haplotype EgCL01; a different haplotype was identified in each of the other five human samples (EgCL02, EgCL03, EgCL12, EgCL13 and EgCL14). **Figure 1 A** also shows the distribution of the haplotypes found in this study; most widespread were the haplotypes EG01 (which was represented in all the regions except for Maule) and the haplotype EgCL01, which was represented in all the regions examined except from isolates from Chiloe Island in Los Lagos region. Out of the 7 samples from dogs analysed from the Coquimbo region, 3 were identified as EG01 while the other 4 were assigned to four different haplotypes (EgCL07, EgCL08, EgCL09 and EgCL10). The further analysis of human samples from this region is necessary to determine the transmission cycle of these haplotypes in this particular region. The haplotype network contains the EG01 haplotype at its centre and reveals a star-like shape (**Figure 1B**). The haplotype EG01 has been previously suggested to be an ancestral haplotype that has spread worldwide after the domestication of animals and migration of human from the Middle East

[8]. Diversity index for haplotypes is 0.875 ±0.032 and for nucleotide diversity a value of 0.000153±0.00017 was obtained, and neutrality indices (D and Fs values) were negative suggesting either some forms of negative selection or population expansion (Table 1). More data from other South American countries is necessary to assess the dispersal and diversity of *E. granulosus s.s.* in the future.

The alignment of the 366bp sections of the sequences from this study with the reference sequence for G1, G2 and G3 identified forty six samples corresponding to G1, 1 to G2 and 7 to G3, while fifteen isolates did not match any of the reference sequences for G1, G2 or G3. Additionally, had the analysis been based on the 827 section of the *cox1* gene used by Boufana and colleagues [12-15] based on previous research [16, 17], many of the new haplotypes that were identified here would have been overlooked. As previously mentioned, there is a clear difference in the outcomes of an investigation of genetic diversity of *E. granulosus s.s.* according to the length of the gene analysed [8, 10]. For example, Casulli *et al* [18] described only 24 haplotypes from 223 samples from Eastern European and 7 haplotypes from 89 samples from Italy, while Andresiuk *et al* [19] reported only 7 *cox1* haplotypes from 69 isolates in Argentina using a short sequence of the *cox1* gene. In the case of the thirteen human samples analysed in this study the method used clearly allowed a deeper understanding of the variability of the parasite found in this host. They were previously described as G1, but now we know that they belong to eight different haplotypes of *E. granulosus s.s*. Autochthonous transmission is likely because the majority of haplotypes (5 of 8) were found both in humans and animals in Chile. Therefore, our study demonstrates the value of using longer sequences, which allow improved resolution of the genetic structure of the parasite population. Data concerning the diversity of CE from South America is abundant especially relating to samples obtained in Argentina. However, it is

mostly based on short sequences of the *cox1* gene [20-24] precluding a comprehensive comparison of the situation in Chile with its neighbour country. On the other hand, previous investigations of *E. granulosus s.s.* haplotypes based on 1,609bp of the *cox1* gene identified only a low diversity in 30 Peruvian samples randomly selected from 57 samples previously examined from different areas in Peru by Moro *et al* [25], describing the presence of only three haplotypes, namely the common haplotype EG01 (16 samples), EG44 (13) and EG43 (1) [8].

Our results challenge the idea that low diversity is a widespread feature of *E. granulosus s.s.* in South America, as the high variability found in this study is unlikely to be limited to Chile. Surprisingly, little is known about the introduction and origin *E. granulosus s.l.* in the Americas. It is assumed that the parasite was introduced into South America from European countries since the arrival of Columbus who brought horses, cattle, sheep, goats and pigs for the first time to the continent. Later settlers continued bringing animals to the newly discovered territories, including dogs which are believed to have played an important role in the colonization [26]. Some of the first animals that arrived at the Americas had originated from Spain while some others were taken aboard on the Canary Islands, which was a common stopover during the transatlantic journey [27]. Interestingly, the Canary Islands were colonized from the fifth century BC by people from North Africa [28] who also brought domestic animals to the island, including goats, sheep, pigs and dogs [27]. If this was the case and some of the animals taken to the new continent were infected with CE, then the origin of some of the parasites that arrived in the Americas could have been not only Spain but also North Africa. The first livestock animals arriving in Chile around 1540 with the Spanish conqueror Pedro de Valdivia included horses and cattle, which were brought from Peru and are thought to have been originated from stocks in Central America [29]. Sheep

arrived later to the Chilean territory, also from Peru, and by 1614 more than six hundred thousand sheep along with 323,956 goats, 39,000 cattle, and 4,278 horses were reported to be kept in the district of Santiago [29]. Considering that most of the founding livestock in Chile were originated in Peru, a deep exploration of *E*. *granulosus* from this neighbour country is necessary to understand the origin of the parasite and how it spread in Chile. Similar studies should be undertaken in Argentina, with which Chile traditionally has had an exchange of livestock animals.

The implications of the microdiversity of *E*. *granulosus s.s*. in the epidemiology and control of the parasite are unclear, and further studies are required to understand if different haplotypes actually differ in biological features. The study of the genetic diversity of *E*. *granulosus s.s.* contributes to the understanding of historical perspectives on the dispersal of the parasite. In the case of this study, our results provide knowledge concerning the parasite's spread in the southern part of the Americas after the arrival of the Spanish colonists, highlighting and confirming the presence of the worldwide spread haplotype EG01 as the main haplotype present in Chile. The high diversity described suggests that the parasite was introduced multiple times from different sources to this country. Further investigations in the microdiversity of *E*. *granulosus s.s*. in Spain, North Africa and other South American countries would be required to elucidate the likely origin of the parasite in this part of the world. The availability of next-generation sequencing technologies can facilitate the sequencing of the full length of the mitochondrial genome of isolates of *E*. *granulosus* from different hosts and geographic areas which will give a deeper understanding of the variability of this parasite.

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Table 1. Nucleotide Diversity and Neutrality indices for the 26 haplotypes described in this study from 69 samples analyzed.

Haplotypes		Diversity		Neutrality	
Samples	Hn	Hd±SD	π±SD	D	Fs
69	26	0.875±0.032	0.00153±0.00017	-201,645	-19,444

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