

## Short Report: Genetic Variation of *Echinococcus canadensis* (G7) in Mexico

Ulises Rodriguez-Prado, Diego Emiliano Jimenez-Gonzalez, Guillermina Avila, Armando E. Gonzalez, Williams Arony Martinez-Flores, Carmen Mondragon de la Peña, Rigoberto Hernandez-Castro, Mirza Romero-Valdovinos, Ana Flisser, Fernando Martinez-Hernandez, Pablo Maravilla,\* and Jose Juan Martinez-Maya

Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autonoma de Mexico, DF, México; Instituto Tecnológico de Milpa Alta, DF, Mexico; Facultad de Medicina, Departamento de Microbiología y Parasitología, Universidad Nacional Autonoma de Mexico, DF, Mexico; Escuela de Medicina Veterinaria, Universidad Mayor de San Marcos, Lima, Peru; Hospital General “Dr. Manuel Gea Gonzalez,” DF, Mexico; Centro de Biología Experimental, Universidad Autonoma de Zacatecas, Zacatecas, Mexico

**Abstract.** We evaluated the genetic variation of *Echinococcus* G7 strain in larval and adult stages using a fragment of the mitochondrial *cox1* gen. Viscera of pigs, bovines, and sheep and fecal samples of dogs were inspected for cystic and canine echinococcosis, respectively; only pigs had hydatid cysts. Bayesian inferences grouped the sequences in an *E. canadensis* G7 cluster, suggesting that, in Mexico, this strain might be mainly present. Additionally, the population genetic and network analysis showed that *E. canadensis* in Mexico is very diverse and has probably been introduced several times from different sources. Finally, a scarce genetic differentiation between G6 (camel strain) and G7 (pig strain) populations was identified.

*Echinococcus granulosus* sensu lato (s.l.) includes species that cause cystic echinococcosis (CE), one of the most important and widespread parasitic zoonoses. Recent phylogenetic studies based on both mitochondrial and nuclear DNA genes show that *E. granulosus* s.l. consists of at least four valid species: *E. granulosus* sensu stricto (s.s.; genotypes G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6–G10). Genotypes G6/G7 are closely related and referred to as camel and pig strains, respectively.<sup>1–3</sup> The pig–dog cycle is mainly present in Mexico and maintains the G7 strain.<sup>4,5</sup> Although there are isolated reports of *E. oligarthrus* in a wild cat,<sup>6</sup> *E. ortleppi* (*E. granulosus* s.l.; G5) in a patient,<sup>7</sup> and *E. granulosus* s.s. (G1) in a rural pig, there is no evidence that these species are maintained in Mexico.<sup>8</sup> No data of CE caused by G7 have been documented in Mexican patients, although there is a high number of *E. canadensis* G7-infected patients in central Europe, pointing to the importance of this strain as a cause of human CE.<sup>9,10</sup> There are only two genetic studies performed in samples from Mexico. Cruz-Reyes and others<sup>5</sup> documented that G7 parasites of Mexican and Polish pig isolates showed similar patterns by restriction fragment length polymorphism (RFLP) of ribosomal DNA (rDNA) internal transcribed spacer 1 (ITS1) and random amplified polymorphic DNA (RAPD) techniques, and although polymerase chain reaction (PCR)-sequencing analysis of mitochondrial *cox1* gen fragment was performed, no polymorphism data were reported. Sharma and others<sup>11</sup> identified two variants (A and B) inside of the G6/G7 group consisting of samples from Mexico and Argentina using five nuclear markers (elongation factor 1 $\alpha$ , transforming growth factor- $\beta$  receptor kinase, thioredoxin peroxidase, calreticulin, and ezrin-radixin-moesin-like protein). Because some local slaughter records from northern Mexico indicate the presence of *Echinococcus* spp. in livestock animals,<sup>5</sup> the objective of this study was to investigate if parasites in pigs and dogs correspond to G7 and if so, describe its genetic variation.

Infected animals were identified in the municipal slaughterhouse of Calera, Zacatecas (north central Mexico), where

farm and backyard livestock animals coming from the whole state and other surrounding states were included. For this purpose, viscera from 387 pigs, 243 bovines, and 32 sheep were inspected for the larval stage of *Echinococcus*. Nine pigs (six pigs from Zacatecas, two pigs from Aguascalientes, and one pig from Morelos) were found infected, and hydatid cysts were obtained under aseptic conditions. After cyst contents were aspirated and centrifuged, aliquots were examined under microscopy to confirm the presence of protoscolices, and pellets were kept in 70% ethanol at –20°C until DNA extraction. Each cyst from each animal was considered as an isolate.

Based on the presence of the parasites previously identified in Calera's slaughterhouse, a rural community located in the central area of Zacatecas at 22°55' N, 102°48' W was selected to look for the adult stage of this parasite. For this search, all dogs (60) present in the community were sampled one time for feces after obtaining verbal consent from the owner; samples were used to identify taeniid eggs by the Faust technique, antigens in stool samples (copro-antigens) by enzyme-linked immunosorbent assay (ELISA; CpAg ELISA), and DNA by Copro-PCR. The CpAg ELISA was performed as described by Allan and others<sup>12</sup> and Moro and others.<sup>13</sup> For Copro-PCR, only positive samples by CpAg ELISA were analyzed using JB3 and JB4 primers to amplify a *cox1* gen fragment.<sup>14</sup> Coprological analysis of dogs showed that 11 samples were positive by CpAg ELISA (18.3%); only 2 of these samples had taeniid tapeworms (3.4%), and 3 of 11 samples yielded products of approximately 450 bp. All amplicons obtained of hydatid cysts and fecal samples were purified, sequenced on both strands, submitted to GenBank (accession numbers KF734649–KF734660), and compared with several mitochondrial DNA sequences of *cox1*. Dogs positive for taeniid eggs or antigens were purged and treated with praziquantel at 30 mg/kg and arecoline bromide at 2 mg/kg. The protocol was previously approved by the Ethics and Research Committees of the General Hospital “Dr. Manuel Gea Gonzalez”; government and health authorities of the municipality and community also authorized our study.

All sequences were subjected to the Basic Local Alignment Search Tool (BLAST) search in the GenBank database; multiple alignments were performed with the CLUSTAL W and MUSCLE programs,<sup>15,16</sup> with manual adjusted in MEGA program v5<sup>17</sup> to determine the appropriate model of molecular

\*Address correspondence to Pablo Maravilla, Hospital General “Dr. Manuel Gea Gonzalez,” Departamento de Ecología de Agentes Patógenos, Mexico, DF, 14080 Mexico. E-mail: maravillap@yahoo.com

TABLE 1  
Genetic differentiation indexes between different paired sequences of *E. canadensis* genotypes obtained from animals

Population A	Population B	$F_{ST}$	$\Phi_{ST}$	AMOVA			References
				SS	VC	Percent	
G6	G7	0.031	0.085	1.640	0.060	8.5	30–38
G6	G8	0.893	0.937	37.767	5.395	93.7	
G6	G10	0.624	0.613	15.798	0.726	61.3	
G7	G8	0.783	0.760	27.250	4.315	76.0	30,31,39,40
G7	G10	0.359	0.336	8.722	0.532	33.6	
G8	G10	0.882	0.881	40.025	5.991	88.1	30,34,36,39
Mexico (G7)	Europe (G7)	0.201	0.179	3.494	0.259	17.9	30,31,40,41
Latin America (G7)	Europe (G7)	0.146	0.113	2.461	0.138	11.3	
Latin America (G7)	Africa (G6)	0.147	0.154	3.334	0.171	15.4	31,33,35
Latin America (G7)	Asia (G6)	0.156	0.126	2.722	0.144	12.6	30,31
Latin America (G7)	Africa–Asia (G6)	0.151	0.205	3.833	0.180	20.6	30,31,33,35
Europe (G7)	Africa (G6)	0.047	0.043	0.727	0.022	4.3	30,33,35,40,41
Europe (G7)	Asia (G6)	0.061	0.019	0.472	0.024	9.1	30,40,41
Europe (G7)	Africa–Asia (G6)	0.042	0.060	0.650	0.233	6.0	30,33,35,40,41

Europe (G7) includes G7 sequences from Italy, Poland, and Romania. Latin America (G7) includes G7 sequences from Mexico and Peru. Africa (G6) includes G6 sequences from Algeria, Ethiopia, Mauritania, and Sudan. Asia (G6) includes G6 sequences from Iran and Kazakhstan. Africa–Asia (G6) includes G6 sequences from China, Iran, Mauritania, Mongolia, and Russia. SS = sum of squared; VC = variance of components.

evolution in the Modeltest 3.7 program.<sup>18</sup> The phylogenetic reconstruction using Bayesian inference was performed with Mr Bayes 3.2.1 program.<sup>19</sup> Unrooted haplotype networks were created using NETWORK 4.611 software and nested according to the rules in median-joining networks.<sup>20</sup> An analysis of genetic diversity within and between populations was performed using DnaSPv4<sup>21</sup> and included nucleotide diversity ( $\pi$ ), haplotype polymorphism ( $\theta$ ), genetic differentiation index ( $F_{ST}$ ), and Tajima's D test. Analysis of molecular variance (AMOVA) was used to examine the population genetic structure between populations by  $\Phi_{ST}$  as the genetic fixation index (analogous to  $F_{ST}$ ) obtained by ARLEQUIN software.<sup>22</sup>

After multiple alignments, all sequences of larval and adult stages showed 98% or higher identity with *E. canadensis*, whereas the Bayesian phylogenetic tree and the haplotype network inference grouped these sequences in the *E. canadensis* G7 cluster. Sequences for *cox1* of *E. canadensis* from Africa, Asia, Europe, Latin America, and North America deposited in the GenBank databases ( $N = 58$ ) as well as our sequences (accession numbers KF734649–KF734660) were analyzed. The results for  $\pi$  and  $\theta$  were 0.0118 and 0.718, and the result of Tajima's D test was  $-2.1885$  ( $P < 0.01$ ). Genetic differentiation indexes between different paired sequences of *E. canadensis* genotypes are shown in Table 1. Globally, the pair between G6 and G7 exhibited the lowest values of  $F_{ST}$  and  $\Phi_{ST}$  (0.03 and 0.08, respectively) as well as a percentage of variation by AMOVA less than 10%. The other genotypes showed values of  $F_{ST}$  and  $\Phi_{ST} \geq 0.3$  with a percentage of variation by AMOVA over 30%. Interestingly, when G6 and G7 were separated by geographic area,  $F_{ST}$  and  $\Phi_{ST}$  values ranged from 0.15 to 0.2 when matched between Latin America and Europe and between Africa and Asia, whereas for matching pairs within Europe, Africa, and Asia,  $F_{ST}$  and  $\Phi_{ST}$  were  $\leq 0.06$ , suggesting that populations from Latin America reflect a great genetic differentiation compared with populations of Europe, Africa, and Asia. We did not find available sequences of G7 in humans; however, there are some sequences for G6 in individuals from Africa (Mauritania) and Asia (China, Iran, and Mongolia) that presented similar  $F_{ST}$  and  $\Phi_{ST}$  values as those sequences for G6 and G7 obtained from livestock.

For the network analysis, haplotypes of *E. canadensis* (G6, G7, G8, and G10), according to their hosts and country of

origin, were included and exhibited three relevant dispersion centers (clustering more than nine haplotypes in each one of them): one for G10 from North America with elk/wolf, one for G6/G7 from Iran, Mauritania, and Peru with camel and sheep, and one for G6/G7 from Africa, Asia, and Latin America with cattle, camel, dog, elk, goat, and human. Interestingly, some G7 pig haplotypes from Mexico are displayed around the third dispersion center; in contrast, other G7 haplotypes from European and Asian countries are clustered around the second dispersion center (Figure 1).

The sequences obtained from three dogs and nine infected pigs showed that *E. canadensis* (G7) was the only strain identified, indicating that it is the main genotype present in Mexico, which had been previously reported.<sup>4,5</sup> This study also shows that *E. canadensis* (G6, G7, G8, and G10) is lightly more polymorphic than other species of the genus *Echinococcus* ( $\pi = 0.0118$ ), and the negative value of Tajima's D test suggests a recent expansion for the populations. Haag and others<sup>23</sup> reported  $\pi = 0.0005$  for *E. multilocularis* and  $\pi = 0.0090$  for *E. granulosus* using mitochondrial (*nad*) and nuclear (*Act11*, *Hbx2*, and *AgB*) sequences; in addition, Sharma and others<sup>11</sup> performed a population genetic analysis of *E. granulosus* s.s. using *cox1* sequences and found that  $\pi$  ranged from 0.0039 to 0.0093 for *E. granulosus* s.s. isolates from India, and they also found a negative value for Tajima's D test. Small sample sizes and lengths of the nucleotide sequences might affect the  $\pi$  values, showing a tendency toward underestimation.<sup>24</sup> In addition, most studies of genetic variation in *Echinococcus* have used around a dozen sequences; therefore,  $\pi$  results might not be directly comparable among them. However, even under these considerations, this comparison allows us to highlight the genetic diversity among populations of *E. canadensis*. Furthermore, we found that, in *E. canadensis* populations, G6 and G7 have a scarce differentiation ( $F_{ST}$  and  $\Phi_{ST}$  close to 0.1), whereas it is high for *E. canadensis* G8 and G10 ( $F_{ST}$  and  $\Phi_{ST} > 0.6$ ). In contrast, in a study focused on the genetic diversity of *E. granulosus* s.s., hydatid cysts from four European countries (Bulgaria, Hungary, Romania, and Italy) were evaluated by sequences of *cox1* and showed  $F_{ST}$  values up to 0.187.<sup>25</sup> In this study, when G6 and G7 were divided in geographic areas, a similar genetic differentiation was observed with  $F_{ST}$  and  $\Phi_{ST} < 0.1$ , except when Latin America (G7) was matched with

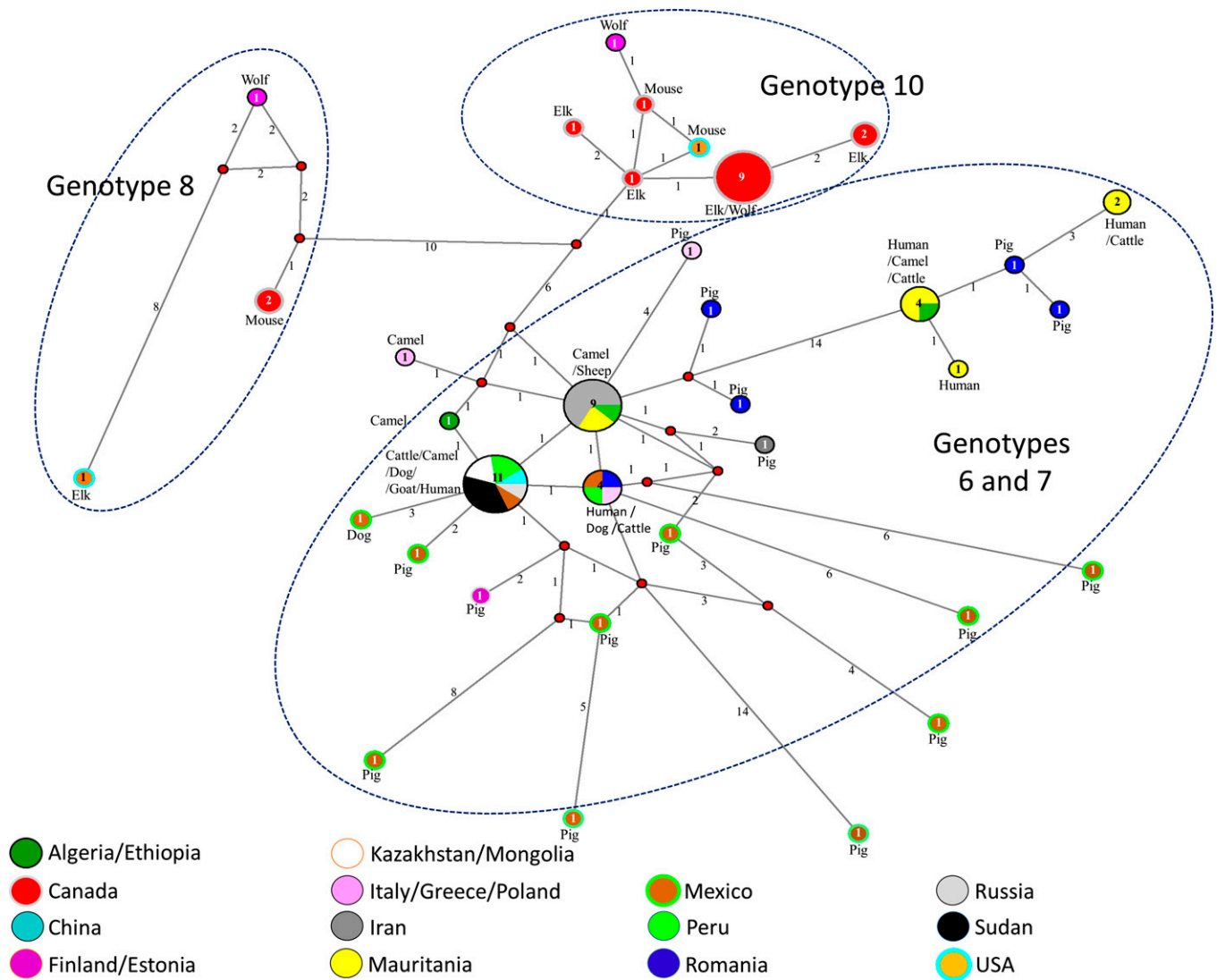


FIGURE 1. Haplotype network for *E. canadensis* using *cox1* sequences of different countries and hosts. Numbers on branches refer to mutational changes. Sizes of circles are proportional to haplotype frequencies (numbers of haplotypes are shown inside circles). Thus, major circles represent ancestral haplotypes, and small circles represent missing haplotypes. Hosts are shown on a side of the haplotypes, and the three big ellipses with discontinuous lines containing G6/G7, G8, and G10.

Europe, Africa, or Asia ( $F_{ST}$  and  $\Phi_{ST}$  = 0.15–0.2), suggesting that the former population reflects a great genetic differentiation regarding the latter populations. This is strengthened by the network analysis, in which some haplotypes of pigs from Mexico are clustered in different branches from those from pigs of European countries.

Based on the network analysis, we might deduce the following inferences. (1) *E. canadensis* G7 in Mexico is very diverse and has probably been introduced from abroad several times from different sources (i.e., Figure 1 shows that six Mexican isolates have from 4 to 14 mutational changes between the isolate and the main haplotype). (2) Haplotypes grouped in the North American wildlife cluster (G10) are closer within them (with one or two mutational changes), and they are placed far away from Mexican isolates; thus, they might be ruled out as sources of introduction to Mexico. (3) Differentiation between G6 and G7 would not make any sense based on the differentiation of genetic indexes found for both genotypes ( $F_{ST}$  and  $\Phi_{ST}$  close to 0.1). Additionally, one of the main

ancestral dispersion centers in the network analysis clustered identical haplotypes of G6 and G7 from China, Mexico, Peru, Sudan, and Russia. The species status of *E. canadensis* is still controversial,<sup>1–3,5,25</sup> because biologically different strains (G6–G10) have been unified. The camel (G6) and pig (G7) strains (both maintained primarily by dog-mediated domestic lifecycles from tropical to temperate zones) are ecologically and geographically segregated from G8 to G10<sup>2,26</sup>; therefore, some works have suggested that G6 and G7 should be treated as a single species: *E. intermedius*.<sup>5,27</sup> However, in recent taxonomic revisions, this proposal has been considered inappropriate,<sup>2,26</sup> and the specific name of *E. canadensis* seems to be the most suitable for handling the closely related genotypes. Thompson and Lymbety<sup>28</sup> have argued that knowledge of the genetic structure of cestodes can be applied to the epidemiology and the control of these parasites, because genetic variation within and between populations determines future evolutionary changes, genetic differentiation, and speciation. According to our results, it is probable that *E. canadensis* G7 has been

accidentally introduced from abroad several times through different sources, except from North America (where G10 is more prevalent). This knowledge may have important implications for control of the zoonosis, mainly in areas that lack adequate veterinary control, which could prompt an important health problem. Although presently there are few cases of human cystic echinococcosis in Mexico, interestingly, a study performed in a rural community where an autochthonous human case of CE was detected in 2006 showed that, although some risk practices (such as feeding dogs with infected viscera) were observed, no data of CE in livestock and canine echinococcosis were found, suggesting that CE in Mexico has an unclear pattern.<sup>29</sup>

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Authors' addresses: Ulises Rodriguez-Prado and Jose Juan Martinez-Maya, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autonoma de Mexico, DF, Mexico, E-mails: amodelmerol123@yahoo.com and jjmm@unam.mx. Diego Emiliano Jimenez-Gonzalez, Instituto Tecnológico de Milpa Alta, Direccion General de Educacion Superior Tecnologica (DGEST), Mexico, DF, Mexico, E-mail: diego\_emiliano\_j@hotmail.com. Guillermina Avila and Ana Flisser, Facultad de Medicina, Departamento de Microbiologia y Parasitologia, Universidad Nacional Autonoma de Mexico, Mexico, DF, Mexico, E-mails: guilleavila2000@yahoo.com and flisser@unam.mx. Armando E. Gonzalez, Escuela de Medicina Veterinaria, Universidad Mayor de San Marcos, Lima, Peru, E-mail: agonzale@jhsph.edu. Williams Arony Martinez-Flores, Rigoberto Hernandez-Castro, Mirza Romero-Valdovinos, Fernando Martinez-Hernandez, and Pablo Maravilla, Hospital General "Dr. Manuel Gea Gonzalez," Departamento de Ecologia de Agentes Patogenos, Mexico, DF, Mexico, E-mails: arony@live.com.mx, rigo37@gmail.com, mirzagrv@yahoo.com, fherxyz@yahoo.com, and maravillap@yahoo.com. Carmen Mondragon de la Peña, Centro de Biología Experimental, Universidad Autonoma de Zacatecas, DF, Mexico, E-mail: hidatidosiszac@hotmail.com.

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