Tropical Biomedicine 34(4): 903–910 (2017)

Prevalence, associated risk factors, and phylogenetic analysis of *Echinococcus granulosus* isolated from free-range Tibetan pigs in Tibet, China

Li, K.[1], Zhang, L.H.[1], Zhang, H.[1], Luo, H.Q.[1], Lan, Y.F.[1], Shahzad, M.[1,3], Wang X.Q.[1,4], Mehmood, K.[1,3] and Li, J.K.[1,2*]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

[2]Laboratory of Detection and Monitoring of Highland Animal Disease, Tibet Agriculture and Animal Husbandry College, Linzhi 860000 Tibet, People's Republic of China

[3]University College of Veterinary & Animal Sciences, The Islamia University of Bahawalpur, Bahawalpur, 631000, Pakistan

[4]DeQing Animal Husbandary and Veterinary Bureau, Huzhou, 313299, People's Republic of China

[*]Corresponding author e-mail: lijk210@sina.com

Received 27 January 2017; received in revised form 20 April 2017; accepted 22 April 2017

Abstract. The current study was performed to investigate the prevalence, associated risk factors exploration and phylogenetic analysis of *Echinococcus granulosus* (E. *granulosus*) genotypes isolated from Tibetan pigs. A total 373 Tibetan pigs were examined during 2014 and 2015, and the variables potentially associated with *E. granulosus* infection were explored with a multivariable logistic regression model. *E. granulosus* cysts (n=37) were collected from Tibetan pigs (lungs or livers). Fragments amplification of mitochondrial (mt) DNA of *cox1* (shorter and longer) and *atp6* were employed. The genotype of *E. granulosus* were identified by sequence and phylogenetic analysis. Results showed the prevalence of *E. granulosus* in Tibetan pigs was 9.9%. The prevalence of *E. granulosus* in male and female Tibetan pigs was 6.8% and 13.3%, with a significant difference in the two genders ($P<0.05$). In different seasons, the infection rate of *E. granulosus* in Tibetan pigs were ranged from 5.8% to 12.3%. *E. granulosus* infection rates in different growing stages of Tibetan pigs were ranged from 4.4% to 15.9%, with a statistical difference in the three stages ($P<0.01$). The prevalence of *E. granulosus* in Tibetan pigs were 7.9% in 2014 and 13.0% in 2015. Genders and growing stages were demonstrated to be risk factors to influence the prevalence significantly through multivariable logistic regression model. All the three fragments were successfully amplified from each of the 37 cysts. *E. granulosus* genotypes of G4 and G6 were identified by comparing with reference sequences of *E. genotypes* available at NCBI database and phylogenetic analysis by using MEGA software.

INTRODUCTION

Echinococcosis is caused by *Echinococcus granulosus* (*E. granulosus*), it is considered a zoonotic disease and spread world-wide (Chaligiannis *et al*., 2015). *E. granulosus* is generally known because of its larval stage, which causes major human health problem and economic losses world-widely (Chaligiannis *et al*., 2015; Zhang *et al*., 2015). The larval stage (hydatid cyst) can parasitize on a number of mammalian intermediate hosts, human beings are recognized as the main reason for its global distribution (Craig *et al*., 2007).

To date, as the high genetic diversity characteristic of *E. granulosus* with genotypes (G1-G10) have been identified (Chaligiannis *et al*., 2015). By revising of the taxonomy of the genus *Echinococcus*, the complex *E. granulosus* are recognized to be composing of four species: *E. granulosus*

sensu stricto (s.s.) (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6-G10) according to the complete mitochondrial (*mt*) genome and nuclear data (Chaligiannis *et al*., 2015; Fadakar *et al*., 2015). The G1 gene-type can not only develop fertile cysts in sheep, but it can also infect other animals including pigs and humans (Monteiro *et al*., 2014; Fadakar *et al*., 2015).

Dogs and canid animals act as the definitive hosts of *E. granulosus* adults, whilst intermediate hosts are swine and considerable number of ungulates including goats, sheep and cattle, harboring the hydatid cyst (Vaniscotte *et al*., 2011). Previous study report that serious problems in Central Asia including China, Kazakhstan, Tajikistan, Kyrgyzstan, Mongolia, Uzbekistan, Afghanistan, Turkmenistan, Iran and Pakistan were caused by Echinococosis (Zhang *et al*., 2015). More than 50% of the native people are exposed, particular farmers and local herds-man are at high risk of *E. granulosus* and *E. multilocularis* infection (Yu *et al*., 2008; Zhang *et al*., 2015).

A Chinese local pig breed with hard black hair named Tibetan pig is mainly perches in the high (more than 3000 m), hypoxic and cold Plateau in Tibetan Plateau and surrounding areas (Zhang *et al*., 2014; Li *et al*., 2016). The typical characteristics of the Tibetan pig are disease resistance, easy breeding and carcass lean high-quality due to free range system employed to the Tibetan pigs and are mainly fed by the combined with the dry lot husbandry (Zhang *et al*., 2014; Li *et al*., 2017). This animal is of great importance to local herdsman in economical (Li *et al*., 2016; Li *et al*., 2017), as the meat of Tibetan pigs are rich in protein, with a tender texture, and high content of amino acids, thus it is an important source of protein for native Tibetans (Wu *et al*., 2012; Zhang *et al*., 2014; Li *et al*., 2017). Therefore, in such areas, any zoonosis disease remains a serious public health threat and causes significant economic losses to native herdsman (Li *et al*., 2014; Li *et al*., 2015).

The infection rate of AE/CE in human has been detected out as high as 9.5% on the Qinghai-Tibetan Plateau and its surrounding regions, especially in Tibetan communities (Zhang *et al*., 2015). However, scarce information is available about the gene characteristic of *E. granulosus* in Tibetan pigs from the high plateau. The current research herein is designed to perform molecular identification of the genotype of *E. granulosus* isolated from Tibetan pigs in Tibet, China for the first time.

MATERIALS AND METHODS

Sample collection and DNA isolation: During 2014 to 2015, the carcass and viscera of 373 Tibetan pigs were inspected to detect the presence and location of hydatid cysts through visual inspection. Hydatid cysts were sorted into fertile, sterile or caseous cysts. Fertility was decided after dissection of the cyst, and aseptically collection of the germinal layer and the protoscoleces was followed. Light microscopy was employed to inspect protoscoleces to determine viabilities (flame cell activity, peristaltic motility together with staining with a 0.2% aqueous eosin solution) as previous research reported (Chaligiannis *et al*., 2015).

A total 37 cysts were obtained from Tibetan pigs livers/lungs. The whole cysts detected were fertile. DNA of *E. granulosus* extraction from protoscoleces (or germinal layer) were carried out by employing commercial DNA extraction reagent kit (Tiangen Biotech Co., Ltd, China) under the guidance of the manufacturer's instructions. The eluted DNA was stored at -20°C for PCR analysis.

Gene amplification and DNA electrophoresis: Fragments of 2 genes of *mt* DNA were amplified using previously reported oligonucleotide primers (Snabel *et al*., 2009). The PCR mixture contained 13µl autoclaved distilled water, 5.5µl PCR Buffer (10×), 2.5µl dNTPs (2 .5 mM), 1µl DNA, 1µl Taq, 1µl of each forward and reverse primer (working concentration: 10 µmol/L) in a 25µl reaction volume. For the cytochrome coxidase subunit 1 (cox1) shorter fragment (~366 bp) (cox1: F: 5'-TTTTTTGGGCATCC TGAGGTTTAT-3', R: 5'-TAAAGAAAGAACAT AATGAAAATG-3'), under the following cyclic

PCR conditions: an initial denaturation step at 95°C for 5 min; 30 cycles at 95°C for 45 sec, 54°C for 40 sec, and 72°C for 50 sec; followed by a final extension step at 72°C for 5 min. The *cox1* longer fragment (~789 bp) (cox1 F: 5'-TTGAATTTGCCACGTTTGAA TGC-3', R: 5'-GAACCTAACGACATAACAT AATGA-3') and ATPase subunit6 (atp6) gene (F: 5'-GCATCAATTTGAAGAGTTGGGGA TAAC-3', R: 5'-CCAAATAATCTATCAACTA CACAACAC-3') were amplified under the following conditions: 35 amplification cycles at 94°C for 45 sec, and 58°C for 45 sec, and 72°C for 50 sec after an initial hot start at 94°C and ending with 72°C each for 10 min. All PCR products were examined on a 1.5% agarose gel stained with ethidium bromide following electrophoresis. A Hi-TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd, China) were employed to purify the PCR electrophoresis products under the guidance of the manufacturer's recommendations. Then the products were sequenced.

Phylogenetic analyses: Multiple alignments were utilized to the 37 *mt* genes of *cox*1 and atp6 sequences, respectively by MEGA (version 6). The *cox*1 and *atp6* sequences were compared and phylogenetic analysis were performed using software of MEGA with the methods of the neighbor-joining algorithm, respectively and the distances were calculated through the Tajima-Nei means with reference sequences of *Echinococcus* genotypes available at NCBI database. The branches stability was assessed after bootstrapping with 1000 replicates. These strains for cox1 were KX039951.1, KU925400.1, AF297617.1, M84662.1, M84663.1, AF346403.1, AB235846.1, AB208063.1, AB235847.1, AB235848.1, AB208064.1, AB745463.1 and AB018440.2. These strains for atp6 were as follows: AB235847.1, AB745463.1, AB235848.1, HG975331.1, HM804589.1, AY056613.1 and AB235846.1.

Statistical analysis: Exploring variables potentially associated with exposure to *E. granulosus* infection was performed by employing a multivariable logistic regression model. Statistically significant between levels within factors and interactions was recognized, when probability (P) value<0.05. Odds-ratios (OR) with 95% confidence intervals (CI) according to the likelihood ratio statistics. Statistical analyses was performed through the IBM SPSS Statistics 20.0 (SPSS Somers, NY).

RESULTS

During slaughter, a considerable number of *cysts* were observed in the lungs or livers in Tibetan pigs (Fig. 1). The overall prevalence of *E. granulosus* in Tibetan pigs was 9.9% (95% CI: 7.1-13.4) (Table 1). The prevalence of *E. granulosus* in male and female Tibetan pigs was 6.8% (95% CI: 3.7-11.3) and 13.3% (95% CI: 8.7-19.1), and a significant difference was discovered in the different genders (P<0.05) (Table 1). In different seasons, the prevalence of *E. granulosus* in Tibetan pigs were ranged from 5.8% to 12.3% (Table 1). The infection rate of *E. granulosus* in different growing stages Tibetan pigs were ranged from 4.4% to 15.9%, and a statistical difference was found in the different stages (P<0.01) as given in Table 1. The infection rates of *E. granulosus* in Tibetan pigs were 7.9% in 2014 and 13.0% in 2015, which demonstrated an increasing trend of *E. granulosus* infection in Tibetan pigs.

In the current results, season was not significant according to conditional stepwise logistic regression (P>0.05), while two factors (genders and growing stages) were demonstrated to be risk factors influencing the prevalence significantly. In different genders, female Tibetan pigs (13.3%) had a double (OR=2.105, 95% CI=1.037-4.273, P<0.05) higher risk of infection by comparison with male Tibetan pigs (6.8%) (Table 2). In different growing stage, adult Tibetan pigs (15.9%) had a four times (OR=4.145, 95% CI=1.399-12.280, P<0.01) higher risk of being positive compared to juvenile Tibetan pigs (4.4%), while no difference (P>0.05) was found in sub adult Tibetan pigs (6.0%) compared to juvenile Tibetan pigs (4.4%) as shown in Table 2.

Figure 1. *E. granulosus* infected livers in Tibetan pigs in Tibet, China.

Table 1. Prevalence of *E. granulosus* in Tibetan pigs in different gender, season and growing stage in Tibet in 2014 and 2015

Variable	Samples	Positive samples	Prevalence (%)	CI (95%)
Gender[a]				
Male	192	13	6.8%	3.7-11.3
Female	181	24	13.3%	8.7-19.1
Season				
Spring	52	3	5.8%	1.2-15.9
Summer	154	19	12.3%	7.6-18.6
Autumn	97	9	9.3%	4.3-16.9
Winter	70	6	8.6%	3.2-17.7
Growing stage[b]				
Juveniles	92	4	4.4%	1.2-10.8
Sub adults	117	7	6.0%	2.4-11.0
Adults	164	26	15.9%	10.6-22.4
Year				
2014	227	18	7.9%	4.8-12.2
2015	146	19	13.0%	8.0-19.6
Total	373	37	9.9%	7.1-13.4

[a]There was a significant difference among the different genders of the prevalence of *E. granulosus* infection in Tibetan pigs in 2014 and 2015. ($p < 0.05$ $\chi 2=4.390$).
[b]There was a significant difference among the different growing stages of the prevalence of *E. granulosus* infection in Tibetan pigs in 2014 and 2015. ($p < 0.01$ $\chi 2=11.688$).

Table 2. Odds ratios for gender and growing stage of Tibetan pigs as risk factors for *E. granulosus* prevalence in Tibetan pigs (n = 373)

Factor	Category	Prevalence (%)	OR	95% CI	P-value
Gender	Male	6.8%	reference		
	Female	13.3%	2.105	1.037-4.273	0.036
Growing stage	Juveniles	4.4%	reference		
	Sub adults	6.0%	1.400	0.397-4.936	0.599
	Adults	15.9%	4.145	1.399-12.280	0.006

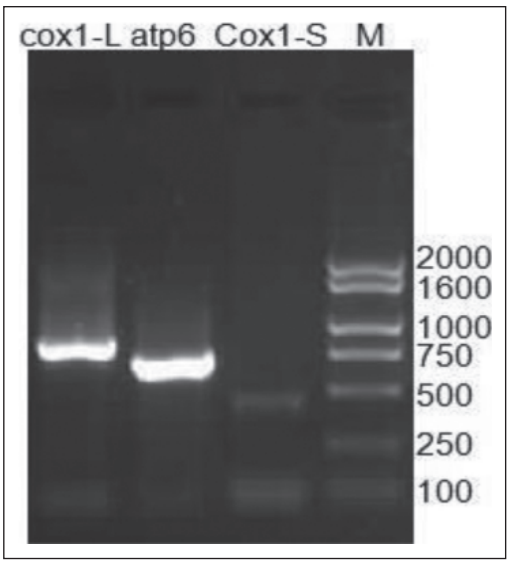

Figure 2. Specific PCR amplification of cox1 shorter (cox1-S), cox1 longer (cox1-L) and atp6 of *E. granulosus* gene on 1.5% agarose gel. Marker: 2000 1600 1000 750 500 250 100 bp DNA ladder.

DISCUSSION

The overall infection rate of *E. granulosus* in Tibetan pigs was 9.9%, which was in accordance with Zhang *et al.* (2015), who stated that *E. granulosus* infection was ranged from 10 to 67% in dogs through necropsy on the Qinghai-Tibet Plateau. The high infection rates revealed a relatively high risk for herdsmen, as Wang *et al.* (2005) reported a relatively high prevalence of 2.7% of cystic echinococcosis in Hobukesar in Xinjiang which is adjacent to Tibet.

In the current research, all the three *mt* genomes of *cox1* (shorter and longer) and *atp6* were amplified successfully from each of the 37 *E. granulosus* cysts collected from Tibetan pigs, respectively. The presented results confirms the *E. granulosus* infection in Tibetan pigs by molecular methods. By employing multiple alignments of the 37 *mt* genomes of *cox*1 (shorter and longer) and *atp6* sequences, respectively by MEGA (version 6), barely evident difference were found in each of the three *mt* genomes among the 37 sequences, respectively. Previously, G1 genotype of *Echinococcus* were reported in cattle, yaks, dogs and humans (de la Rue *et al.*, 2011; Balbinotti *et al.*, 2012; Hu *et al.*, 2015). By comparing and phylogenetic analysis of *E. granulosus mt* genome of *cox*1 and *atp6* sequences with reference sequences of *Echinococcus* genotypes available at NCBI database indicated that the presented cysts were demonstrated to be G4 and G6 (Fig. 3 & 4). The results presented herein were not in accordance with previous study that *E. granulosus* were identified as genotype G1 and G6 from sheep, human and yaks inhabit on the Qinghai-Tibetan plateau by DNA marker of *mt* DNA of *cox2* (Hu *et al.*, 2015). While, Sanchez *et al.* (2012) and Monteiro *et al.* (2014) reported genotypes (G1 and G7) isolated from swine in Peru and Brazil, respectively. These results obviously revealed of the susceptibility of swine to multiple geno-type of *Echinococcus* (Eckert and Thompson, 1997). The Tibetan pigs were demonstrated as potential host of this zoonosis.

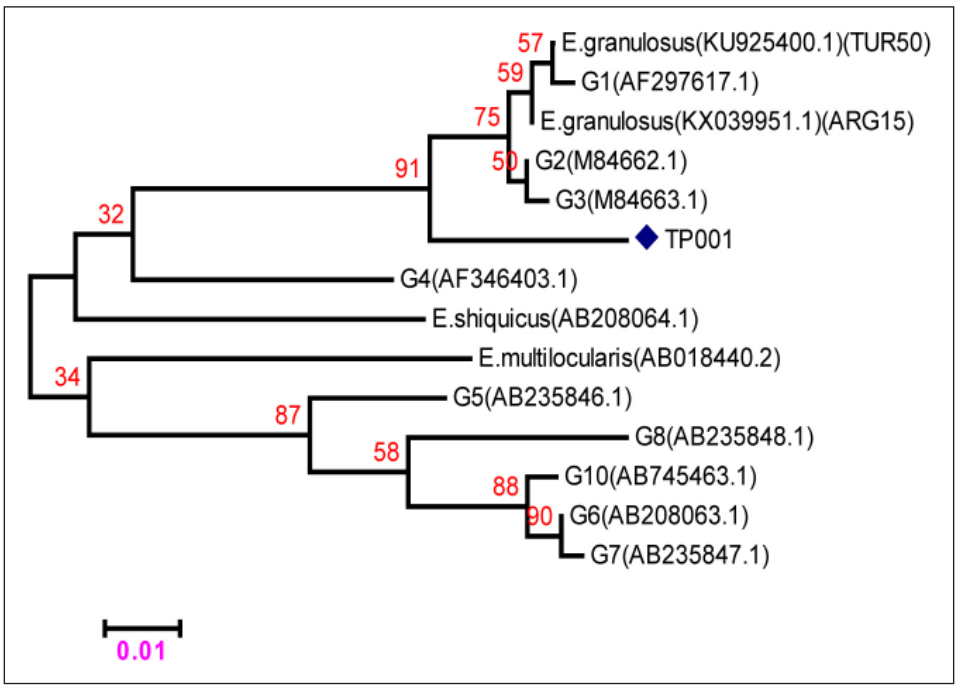

Figure 3. Neighbor joining (NJ) tree based on cytochrome c oxidase I gene (cox-I) (longer) for *E. granulosus*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown. Phylogenetic analyses were conducted in MEGA6.

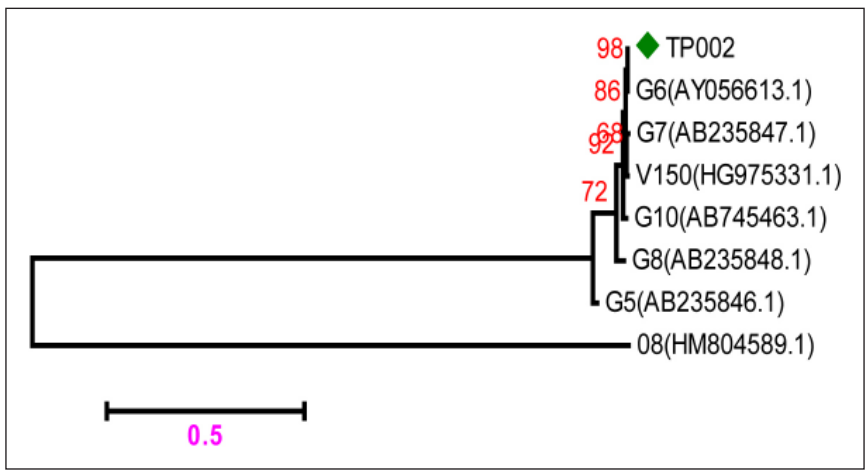

Figure 4. Neighbor joining (NJ) tree based on cytochrome ATPase subunit6 (atp6) for *E. granulosus*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown. Phylogenetic analyses were conducted in MEGA6.

In china, *E. granulosus* has been reported in 27 provinces and mainly found in western and northwestern regions (Hu *et al.*, 2015), and tremendous economic losses by *E. granulosus* consider it one of the most important parasites (Hu *et al.*, 2015). A prevalence of human AE/CE ranging from 0.4% to 9.5% was uncovered in Tibetans and

the prevalence of herdsmen / pastoralists were significant higher in that areas (Zhang *et al*., 2015). The reason may because of that dogs play an important role as prevalence ranged 3-23% were reported of infection with AE on the Qinghai-Tibetan Plateau (Zhang *et al*., 2015); 11.3% *Echinococcus* infection in pikas and 15% *E. multilocularis* infection in Tibetan (Han *et al*., 2009; Vaniscotte *et al*., 2011). The current results of *E. granulosus* genotype G1 and G6 from Tibetan pigs may also contribute to the transmission of AE on the Qinghai-Tibet Plateau.

In conclusion, a widely acknowledgment human pathogens (Amer *et al*., 2015), *Echinococcus* genotypes of G4 and G6 were identified by *mt* genome of *cox*1 and *atp6* isolated from cysts of lungs or livers in Tibetan pigs on the high and remote plateau for the first time. The overall prevalence of *E. granulosus* in Tibetan pigs was 9.9%, genders and growing stages were revealed to be risk factors influencing the prevalence significantly. A significant public health concern should be aroused for the potential risk of *E. granulosus* transmitted to native people and other animals, and economically important for animal production.

Conflict of interest: The authors state that they is no competing interests.

Acknowledgements. The current research was supported by Key Science Fund of Science and Technology Agency of Tibet Autonomous Region and projects in the National Science & Technology Pillar Program during the 12th Five-year Plan Period (2012BAD3B03).

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