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Short Communication

Microvariant *coxI* Gene of *Echinococcus granulosus* Sensu Stricto Cysts Found in One Sheep Liver with Two Million of Protoscoleces

Gang Guo[1,2], Wenjing Qi[1,3], Baoping Guo[1], Tian Wang[4] and Jun Li[1]*

[1]*State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medicine Institute, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054, China*
[2]*Suzhou Center for Disease Control and Prevention, Suzhou, Jiangsu 215004, China*
[3]*Basic Medical College, Xinjiang Medical University, Urumqi, Xinjiang 830011, China*
[4]*College of Animal Science, Tarim University, Alar, Xinjiang 843300, China*

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Authors' Contribution
GG, and JL presented the concept, wrote and edited the mauscript. WQ and BG performed sample collection and experiments. TW interpreted the data, and reviewed the mauscript.

Key words
***Echinococcus granulosus*, Microvariant, Cytochrome coxidase subunit I, Protoscoleces**

ABSTRACT

Echinococcus granulosus (Batsch, 1786) is a zoonotic agent and sheep is an important intermediate host in the lifecycle of cystic echinococcosis (CE). Above 2 million of protoscoleces were collected and counted by means of microscopic examination from one sheep liver which was derived from Urumqi, Xinjiang. To determine the genotypes of the parasite, DNA was extracted from germinal layers which had been washed with 1×PBS, isolated randomly from 12 independent hydatid cysts in the sheep liver respectively and used as templates to amplify the partial nucleotide sequences from mitochondrial NADH dehydrogenase 5 (*nad5*) and cytochrome coxidase subunit I (*coxI*) by polymrase chain reaction (PCR) assay. The PCR products were sequenced and analyzed further and G1 genotype of *E. granulosus sensu stricto* (*s. s.*) was confirmed. Furthermore, two microvariants of *coxI* gene were exhibited among 12 cysts. Our finding indicates that the biological capacity of the host for harboring *E. granulosus s. s.* is huge and likely that impacts the transmission of CE that may result in difficulty in terms of the control of CE to some extent. By genotype analysis, it suggests that G1 genotype of *E. granulosus s. s.* with different genetic microvariants can exit in a single sheep liver and may be responsible for the complexcity of vaccination developing, diagnostic tests and drug therapy on CE.

Cystic echinococcosis (CE) is an important parasitic zoonosis which was caused by the larval stage of *Echinococcus granulosus* (Batsch, 1786) *Sensu lato* reported in all continents with the exception of Antarctica (Eckert and Deplazes, 2004). The severe disease is a significant public health issue globally, especially in western China with the peaks of 12% prevalence and annual incidence of 80/100,000 in human in certain communities of Xinjiang or Qinghai in China where up to 99 % of sheep are infected (Craig *et al*., 2007). In addition to the serious impact on the human health, infections in cattle and sheep also caused huge economic losses (Budke, 2006).

Currently, there are 10 recognizable genotypes (G1 to G10) with the DNA sequencing application of mitochondrial cytochrome C oxidase subunit I (*coxI*) and NADH deydrogenase I (*nadI*) gene and 5 species worldwide including *E. granulosus sensu stricto* (*s. s.,* G1-G3*)*, *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6-G10) and *E. felidis* (Vuitton, 2020). In Xinjiang, the genotypes of G1 and G3 were found in sheep, human and rural dogs (Guo *et al*., 2019; Bart *et al*., 2006). G6 genotype (Camel strain) was found in human, *Camelus bactrianus* and rural dogs (Chai *et al*., 1998; Zhang *et al*., 2005). Nevertheless, G1 was the most widely distributed genotype in Xinjiang, China and the world, which was responsible for the majority of human CE cases around the world (Romig *et al*., 2015; Alvarez *et al*., 2014).

Materials and methods

A liver was collected from a Kazakh sheep, female, 4

* Corresponding author: 1742712944@qq.com
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years old by teeth age, carcass weight of above 18 kg, which containing a large number of echinococcal cysts from a slaughterhouse in Urumqi, Xinjiang of China in Autumn 2018. More than 80% of liver volume was taken up by the cysts. We aspirated the cyst fluid containing protosocleces (metacestode, a larval stage of the parasite) and removing cyst membranes carefully with aseptic operations. After mixing protosocleces sediment with 1×PBS, 10 uL of the suspension was taken and placed on a slide for microscope counting. Repeat the aspiration for 3 times and take the average. Then the number of protosocleces sediment per mL was estimated. The protosocleces were observed with microscope after stained with 0.1% methylene blue.

A total of 12 independent hydatid inner cysts were randomly selected from the sheep liver and DNA was extracted from germinal layers of each internal capsule which had been washed thoroughly with 1×PBS, respectively. Whereafter, the NADH dehydrogenase 5 (*nad5*) gene fragment (680 bp) was amplified and analyzing for identifying the differentiation of genotypes G1 and G3 with reference (Kinkar *et al*., 2018). Sequencing of the mitochondrial *coxI* fragment (366 bp) were performed and compared with the published *coxI* sequences of G1 (accesion M84661) (Bowles *et al*., 1992) and the microvariants of the G1 genotype [G1A, G1B, G1C, G1D, G1E, accesions AF458871/72/73/74/75 (Kamenetzky *et al*., 2002) and G1[1], G1[2], G1[3], G1[4], accesions EF393619, EF595654, EU178103/04 (Vural *et al*., 2008)] from *E. granulosus s. s.* as references using Clustal W tools and BioEdit version 5.

Results and discussion

The precipitation volume of protosocleces collected was 9.6 mL containing about 2.02 millions of protosocleces according to our calculation (210,000/mL). It showed 97% of them were viable by dye and observation with microscope. In our previous studies, we generally collected 0-2 mL of *E. granulosus* protosocleces precipitation. An average of about 1 mL protosocleces were from cysts in one contaminated sheep liver, and as we are aware, this is a record number in one sheep liver. Even if the liver is occupied in more than 80% of the volume by cysts of *E. granulosus*, the sheep is still alive. Presence of viable metacestode in sheeps may last several years, until animal slaughtering, and the metacestode burden, both in terms of total mass and number, increases with sheep age (Torgerson *et al*., 2009).

E. granulosus is cycling between an intermediate host such as sheep or cattle/ yark and a definite host such as dog, fox and wolf (Wang *et al*., 2014, 2015; Li *et al*., 2015). Most forms of human CE transmitted in domestic life cycles are involving livestock and dogs. The most known is the sheep-dog cycle, when dogs eat sheep livers or lungs containing echinococcal cysts, protosocleces can develop mature adult tape-worms in dogs' intestines between 40 and 48 days. These worms in dogs' release eggs containing fully developed oncospheres, which can develop echinococcal cysts in intermediate hosts including humans and many species of herbivore animals. If a cyst contains a large number of protosocleces was eaten by one dog, there will be a large number of adult tape-worms in the dog's intestine. In fact, the capacity of worm burden was reported with over 300, 000 *E. granulosus* worms (39% gravid) were collected from a wild dog in central New South Wales, Australia (Jenkins and Morris, 1991). By experimentally infecting dogs, we ever collected 310,000 *E. granulosus* worms from one dog 45 days post-infection, which was challenged with 480, 000 protoscoleces (Zhang *et al*., 2006). A huge number of worms (>300,000) may parasitize in the small intestine if the liver contains 2 million of protoscoleces was swallowed by one dog.

The biological carrying capacity of both intermediate host and definitive host for harboring *E. granulosus* is huge, that may impact the natural transmission of CE. Both *E. granulosus* high infection rate of the visceras in the intermediate hosts and the large number of protoscoleces in each liver will all attribute to CE transmission. Therefore, in general, the number and viability of the protoscoleces in the intermediate host, the number and development degree of the adult worm in the terminal host are two pivotal factors of environmental contamination stress, which presents challenges for public health and safety. So, it is necessary that the multiple-targets control programs should be done to fight against CE to break the parasitic cycle (Wang *et al*., 2014). For intermediate hosts, appropriate administrations of livestock movements and slaughters, vaccination should be done regularly (Torgerson and Heath, 2003; Lightowlers, 2006). For definitive hosts, some measures including the control of owned dog and stray dog (Johansen and Penrith, 2009), prevention the dogs from having access to the contaminated viscera and treatment of dogs with praziquantel should be strengthened (Larrieu and Zanini, 2012).

DNA samples from 12 cysts ranging from approximately 1-7cm in diameter and the G1 genotype of *E. granulosus s.s.* was identified with 759 bp base sequences of the nad5 gene fragment by PCR product sequencing and nucleotide sequences alignment, and 2 different mutation types of the G1 genotype were identified with 366 bp base sequences of the *coxI* gene fragment, which were named G1_1-9 and G1_10-12 deposited in the GenBank with accession number MK732917 and MK732918, respectively. The nucleotide sequences of both G1_1-9 and G1[4] were identical, and G1_10-12 is a

new mutation type because of a mutation G to A at 360 position of the amplified fragment (Supplementary Fig. 1). Amino acid sequence of G1_1-9 deduced has a Valine rather than Alanine at position 19 like G1[4] (Supplementary Fig. 2) and G to A at 360 position of G1_10-12 is a nonsense mutation. The mutation type G1_1-9 is the major type which newly infected the sheep. This finding of 2 distinct *coxI* gene mutation type in one sheep's liver can be explained that the sheep had ingested the eggs excreted by the adut worms with mutant genes. It indicated that the genotype or microvarient of *E. granulosus* was determined just by one cyst or protoscoleces inside from a sheep liver is insufficient according to the previous research outcomes. On the other hand, gene variant of the worm in one host which may lead to different biological characteristics is maybe responsible for the complexity of individual diagnosis, treatment, and vaccination for CE.

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IRB approval

The study was approved by the First Affiliated Hospital OF Xinjiang Medical University, Urumqi, Xinjiang 830054, China.

Ethical approval

The First Affiliated Hospital of Xinjiang Medical University ethical review assessment indicated that ethical review was not required.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230417130448

Statement of conflict of interest

The authors have declared no conflict of interest.

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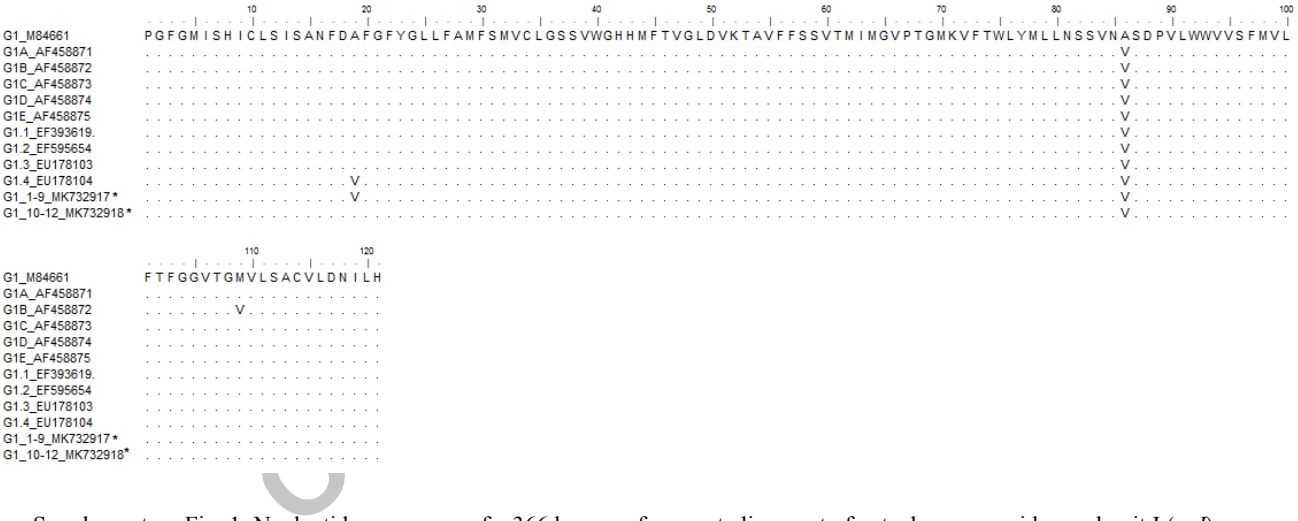
Supplementary Material

Microvariant *coxI* Gene of *Echinococcus granulosus* Sensu Stricto Cysts Found in One Sheep Liver with Two Million of Protoscoleces

Gang Guo[1,2], Wenjing Qi[1,3], Baoping Guo[1], Tian Wang[4] and Jun Li[1]*

[1]*State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medicine Institute, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054, China*
[2]*Suzhou Center for Disease Control and Prevention, Suzhou, Jiangsu 215004, China*
[3]*Basic Medical College, Xinjiang Medical University, Urumqi, Xinjiang 830011, China*
[4]*College of Animal Science, Tarim University, Alar, Xinjiang 843300, China*



Supplementary Fig. 1. Nucleotide sequences of a 366 bp gene fragment alignment of cytochrome c oxidase subunit I (*coI*) gene. The sequences in our study were G1_1-9 and G1_10-12 and reference sequences for the *coI* gene and the genotype G1 variant are shown at the top of them. Dots denote homology with the G1_M84661 sequence.

* Corresponding author: 1742712944@qq.com
0030-9923/2024/0001-0001 $ 9.00/0

Supplementary Fig. 2. Amino acid deduced alignment of cytochrome C oxidase subunit 1 (*coI*) gene. A dot indicates an amino acid that is conserved relative to the G1_M84661 sequence.