

AMCoR

Asahikawa Medical College Repository <http://amcor.asahikawa-med.ac.jp/>

American Journal of Tropical Medicine and Hygiene (2006. Aug) 75
(2):292–294.

Dual infection of animal hosts with different Echinococcus species in the eastern Qinghai–Tibet plateau region of China

Xiao, Ning ; Nakao, Minoru ; Qiu, Jiamin ; Budke, Christine M. ; Giraudoux, Patrick ; Craig, Philip S. ; Ito, Akira

SHORT REPORT: DUAL INFECTION OF ANIMAL HOSTS WITH DIFFERENT *ECHINOCOCCUS* SPECIES IN THE EASTERN QINGHAI-TIBET PLATEAU REGION OF CHINA

NING XIAO, MINORU NAKAO, JIAMIN QIU, CHRISTINE M. BUDKE, PATRICK GIRAUDOUX, PHILIP S. CRAIG, AND AKIRA ITO*

Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan; Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu, Sichuan, China; Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas; LBE, Université de Franche-Comté, Besançon Cedex, France; Cestode Zoonoses Research Group, Biomedical Sciences Research Institute and School of Environment and Life Sciences, University of Salford, Greater Manchester, United Kingdom

Abstract. The eastern Qinghai-Tibet plateau of China is a highly endemic region of echinococcosis where *Echinococcus granulosus* sensu stricto (sheep strain), *Echinococcus multilocularis*, and *Echinococcus shiquicus* are distributed sympatrically. We developed a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method for the identification of the three species in this region. The PCR-RFLP showed the dual infection of animals with different *Echinococcus* spp. The first case was a domestic dog concurrently infected with adults of *E. granulosus* and *E. multilocularis*. The second case was a plateau pika (*Ochotona curzoniae*) harboring metacestodes of *E. multilocularis* and *E. shiquicus* in the liver. The high susceptibility of some mammalian hosts to the parasites and the high prevalence of the three co-endemic species probably increase the chance of mixed infections in the eastern Tibetan plateau.

The three taeniid tapeworms, *Echinococcus granulosus* sensu stricto (sheep strain), *Echinococcus multilocularis*, and *Echinococcus shiquicus*, are distributed in the Qinghai-Tibet plateau of western China.^{1–3} The former two species are the causes of cystic echinococcosis (CE) and alveolar echinococcosis (AE) to humans, respectively, whereas the latter is a newly described species whose pathogenicity, if any, is still unknown.^{3,4} On the eastern part of the Tibetan plateau, various mammals are involved in the two host transmission cycles of *Echinococcus* spp.^{5,6} Dogs, red foxes (*Vulpes vulpes*), and Tibetan foxes (*Vulpes ferrilata*) can serve as definitive hosts, whereas sheep, yaks, Tibetan hare (*Lepus oiostolus*), pika (*Ochotona* spp.), and voles (e.g., *Microtus* spp.) may act as intermediate hosts. Because of the severe environment/weather of high altitude steppe, semi-nomadic pastoralism, entrenched poverty, and traditional customs and beliefs, the Tibetan lifestyle usually results in close contact with both domestic animals and wildlife. Zoonotic diseases will be expected to have a higher prevalence in such communities. Strong religious beliefs, furthermore, do not easily allow for the elimination of stray dogs. These ecological and social factors contribute to the high prevalence and disease burden of both CE and AE in the inhabitants of the plateau.^{4,7}

Previous epidemiologic studies in Shiqu County of Sichuan Province, China, situated on the eastern Tibetan plateau (32°19'–34°20' N, 97°20'–99°15' E), showed that domestic and stray dogs play a key role in the transmission of both *E. granulosus* and *E. multilocularis* to humans.^{7,8} Domestic dogs commonly feed on the offal of slaughtered sheep and other livestock with increased risk of infection with *E. granulosus*. Moreover, dogs allowed to roam free are at risk of infection with *E. multilocularis* when they prey on small mammals such as hares, pikas, and voles that live in the periphery of human communities.^{9,10} Necropsy and purgation surveys of dogs indicated that the prevalence of echinococcal infections fluctuated between 8.4 and 13.2% for *E. granulosus* and between

12.1 and 17.0% for *E. multilocularis*.^{5,9} Despite these high prevalences in dogs, no cases of mixed infections were documented in definitive or intermediate hosts until recently. During the course of canine purgation studies using arecoline hydrobromide, we noticed that some dogs seemed to harbor adult tapeworms of both *E. granulosus* and *E. multilocularis*.⁹ Furthermore, we found both unilocular and alveolar metacestodes in the liver of a plateau pika (*Ochotona curzoniae*). In this study, the polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) was developed to differentiate among *E. granulosus*, *E. multilocularis*, and *E. shiquicus* on the plateau. The PCR-RFLP method showed the dual infections of animal hosts with different *Echinococcus* spp.

Genomic DNA was purified from each metacestode lesion using DNeasy tissue kit (Qiagen, Hilden, Germany). Alkaline-lysates from individual adult tapeworms were prepared as described previously.¹¹ The adult lysate or the metacestode DNA was used as a template for PCR. The primer pair, Ech-LSU/F (5'-GGTTTATTTGCCTTTTGCATCATGC-3') and Ech-LSU/R (5'-ATCACGTCAAACCATTCAAACAAGC-3'), was used to amplify an ~570-bp DNA fragment of mitochondrial gene (*rrnL*, large subunit of ribosomal RNA) containing a species-specific *SspI* restriction site (Figure 1). These primers were designed from the conserved regions of *rrnL* sequences among *E. granulosus* sensu stricto (accession no. AF297617), *E. multilocularis* (AB018440), and *E. shiquicus* (AB159140). PCR amplification was performed in a 25- μ L reaction mixture containing 1 μ L of templates, 200 μ mol/L of each dNTP, 0.2 μ mol/L of each primer, 0.5 unit of *Taq* polymerase (*ExTaq* Hot Start Version; TaKaRa Biomedicals, Tokyo, Japan), and the manufacturer-supplied buffer. Thermal reactions were performed for 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds. Without DNA purification, the amplicons were cleaved with *SspI* (New England BioLabs, Ipswich, MA) at 37°C for 2 hours. The restriction fragments were electrophoresed in 2% agarose gel and stained with ethidium bromide.

A total of 69 Tibetan *Echinococcus* isolates collected from Sichuan, Qinghai, and Gansu Province of China (5 adults and

* Address correspondence to Akira Ito, Department of Parasitology, Asahikawa Medical College, Midorigaoka Higashi 2-1, Asahikawa 078-8510, Japan. E-mail: akiraito@asahikawa-med.ac.jp

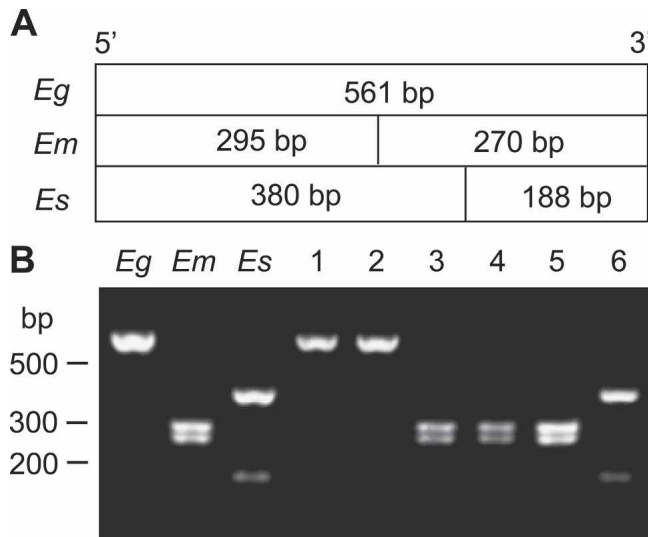


FIGURE 1. PCR-RFLP method to differentiate among *E. granulosus sensu stricto* (Eg), *E. multilocularis* (Em), and *E. shiquicus* (Es). The mitochondrial DNA fragment of *rrnL* was amplified and cleaved with *Ssp* I. **A**, *Ssp* I-restriction maps of the target DNA. **B**, A profile of electrophoresis. The restriction patterns of Lanes 1 to 4 (adult samples from a dog) and Lanes 5 and 6 (larval samples from a pika) showed the dual infection of animals with different *Echinococcus* spp.

21 larvae of *E. granulosus sensu stricto*, 8 adults and 12 larvae of *E. multilocularis*, and 13 adults and 10 larvae of *E. shiquicus*) were used to evaluate the usefulness of the PCR-RFLP method. As reported previously,³ the mitochondrial DNA fragments of cytochrome *b* (*cob*) were amplified and sequenced to verify the species identification. The results of the PCR-RFLP method were completely consistent with those of the *cob* sequencing. The PCR-RFLP method, therefore, seemed to be applicable to epidemiologic surveys in the endemic areas of Tibetan plateau.

To confirm the dual infection of animals with different *Echinococcus* spp. in Shiqu County of Sichuan Province, 65 adult tapeworms from a 2-year-old domestic dog and two hepatic lesions from a plateau pika were subjected to the PCR-RFLP method. One of the hepatic lesions was of unilocular appearance and the other was alveolar. Both lesions in the liver of the pika were separated by normal hepatic tissue. Of 65 adult worms from the dog, 18 (27.7%) were identified as *E. granulosus sensu stricto* and 47 (72.3%) as *E. multilocularis*. The hepatic alveolar lesion in the plateau pika was identified as *E. multilocularis*, but the unilocular lesion in the same liver was identified as *E. shiquicus* (Figure 1). Four amplicons (i.e., two adults identified as *E. granulosus* and *E. multilocularis* and two metacestodes as *E. multilocularis* and *E. shiquicus*) were directly sequenced to confirm the species identification. The resultant *rrnL* sequences proved the PCR-RFLP typing to be correct.

In this study, we showed that mixed infection of mammals with different *Echinococcus* spp. naturally occurred in Shiqu County. This complements other observations in humans in Shiqu, in which ultrasound imaging and serology indicated that a Tibetan patient suffered from both CE and AE.² In the eastern part of the Qinghai-Tibet plateau, diverse assemblages of wild and domestic mammals, and the high susceptibility of host species together with the high prevalences of the parasites probably increase the likelihood of dual infec-

tions of both definitive and intermediate hosts. It seems likely that the dual infection of dogs with *E. granulosus* and *E. multilocularis* may occur because roaming dogs are able to feed on discarded sheep offal containing larval *E. granulosus* and also prey on small mammals that could harbour larval *E. multilocularis*. Surprisingly, however, dual infection of dogs has until now not been reported on the Tibetan plateau. Possible misidentification of immature *Echinococcus* tapeworms should be considered in relation to past and for future morphologic based studies. We also assume that intestinal concomitant immunity eliminates counterpart species when re-infection with the counterpart occurs in dogs. In our survey area, recent microscopical studies on purged tapeworms showed that 2 (0.5%) of 371 dogs were concurrently infected with *E. granulosus* and *E. multilocularis* (C. Budke and others, unpublished data). Dual infection of dogs with both species of *Echinococcus* has also been confirmed in Kazakhstan.¹² Experimental infection of dogs, simultaneously infected with *E. granulosus* and *E. multilocularis*, showed that both parasites develop normally into mature adults and co-exist in the small intestine.¹³ In this case, *E. granulosus* occupied the upper third of the gut, whereas *E. multilocularis* occurred more in the lower third.

The natural infection of a plateau pika with both *E. multilocularis* and *E. shiquicus* leads us to speculate that the dual infection may also occur in definitive hosts, particularly in red fox and Tibetan fox, because both canids seem to prey on the plateau pika (P. Giraudoux and F. Raoul, unpublished data). *E. shiquicus* is quite similar to *E. multilocularis* in its adult stage.³ Therefore, we must consider the possibility that past morphologic identification of the *E. shiquicus* as *E. multilocularis* has probably occurred. The PCR-RFLP method developed in this study is a powerful tool for the accurate identification of *Echinococcus* spp., particularly in the endemic areas of the Tibetan plateau. For the epizootiological surveillance of echinococcosis, we will apply this method to the detection and identification of copro-DNA from Tibetan canids.

In northwestern China, *Echinococcus* genotype G6 (camel strain) is distributed together with *E. granulosus sensu stricto* and *E. multilocularis*.¹⁴ The partial *rrnL* gene of the genotype G6 could be amplified using the present primer set of Ech-LSU/F and Ech-LSU/R; however, it was difficult to differentiate between the genotype G6 and *E. granulosus sensu stricto* because both *Echinococcus* lacked *Ssp*I restriction sites inside the target gene fragment (M. Nakao and N. Xiao, unpublished data). The subsequent sequence analysis of the target gene revealed that a *Bgl*II restriction site is specific to the G6 genotype. In the endemic areas of northwestern China, an additional cleavage with *Bgl* II is necessary for the PCR-RFLP method to differentiate the genotype G6 from *E. granulosus sensu stricto*.

Received December 19, 2005. Accepted for publication April 1, 2006.

Acknowledgments: We appreciate contributions from Wen Yang, Tiaoying Li, and Xingwang Chen, Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention of China; Dalun Liu, Shiqu County Center for Disease Control and Prevention of China; Francis Raoul, Université de Franche-Comté, France; Paul Torgerson, Institute of Parasitology, University of Zürich, Switzerland; and Kenichi Takahashi, Hokkaido Institute of Public Health, Japan.

Financial support: This study was supported by the National Institutes of Health, Ecology of Infectious Diseases Program (1R01 TW01565-01; Principal Investigator, P.S.C.), a Grant-in-Aid from the Japan Society for Promotion of Science to A.I. (14256001, 17256002), and National Nature and Science Fund of China to J.Q. (39070733, 39730400).

Authors' addresses: Ning Xiao, Minoru Nakao, and Akira Ito, Department of Parasitology, Asahikawa Medical College, Midorigaoka Higashi 2-1-1-1, Asahikawa, 078-8510 Hokkaido, Japan, E-mails: xiaoning@asahikawa-med.ac.jp, nakao@asahikawa-med.ac.jp, and akiraito@asahikawa-med.ac.jp. Jiamin Qiu, Department of Echinococcosis and Cysticercosis Control, Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu 610041, Sichuan Province, China, E-mail: qiujiamin45@163.com. Christine M. Budke, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4458, E-mail: Cbudke@cvm.tamu.edu. Patrick Giraudoux, LBE, Université de Franche-Comté, 1 place Leclerc, 25030 Besançon cedex, France, E-mail: Patrick.giraudoux@univ-fcomte.fr. Philip S. Craig, Cestode Zoonoses Research Group, Biomedical Sciences Research Institute and School of Environment and Life Sciences, University of Salford, Greater Manchester M5 4WT, UK, E-mail: p.s.craig@salford.ac.uk.

REFERENCES

1. Qiu JM, Chen XW, Ren M, Luo CX, Liu DL, Liu XT, He DL, 1995. Epidemiological study on alveolar hydatid disease in Qinghai-Xizang plateau. *J Pract Parasit Dis* 3: 106–109.
2. Qiu JM, Lu FJ, Schantz PM, Ito A, Delker C, He JG, Zhang Y, Chen XW, 2000. Epidemiological study on human hydatidosis in Tibetan region of western Sichuan. *Chin J Zoonoses* 16: 77–80.
3. Xiao N, Qiu JM, Nakao M, Li TY, Yang W, Chen XW, Schantz PM, Craig PS, Ito A, 2005. *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika in China. *Int J Parasitol* 35: 693–701.
4. Li TY, Qiu JM, Yang W, Craig PS, Chen XW, Ito A, Giraudoux P, Mamuti W, Yu W, Schantz PM, 2005. Echinococcosis in Tibetan populations, western Sichuan Province, China. *Emerg Infect Dis* 11: 1866–1873.
5. He JG, Qiu JM, Liu FJ, Chen XW, Liu DL, Chen WD, Zhang Y, Schantz PM, 2000. Epidemiological survey on hydatidosis in Tibetan region of western Sichuan II. Infection situation among domestic and wild animals. *Chin J Zoonoses* 16: 62–65.
6. Xiao N, Li TY, Qiu JM, Nakao M, Chen XW, Nakaya K, Yamasaki H, Schantz PM, Craig PS, Ito A, 2004. The Tibetan hare *Lepus oiostolus*: a novel intermediate host for *Echinococcus multilocularis*. *Parasitol Res* 92: 352–353.
7. Wang Q, Qiu JM, Schantz PM, He JG, Ito A, Liu FJ, 2001. Investigation of risk factors for development of human hydatidosis among households raising livestock in Tibetan areas of western Sichuan Province. *Chin J Parasitol Parasit* 19: 93–96.
8. Budke CM, Qiu JM, Craig PS, Torgerson PR, 2005. Modeling the transmission of *Echinococcus granulosus* and *Echinococcus multilocularis* in dogs for a high endemic region of the Tibetan plateau. *Int J Parasitol* 35: 163–170.
9. Budke CM, Campos-Ponce M, Wang Q, Torgerson PR, 2005. A canine purgation study and risk factor analysis for echinococcosis in a high endemic region of the Tibetan plateau. *Vet Parasitol* 127: 43–49.
10. Schantz PM, Wang H, Qiu JM, Liu FJ, Saito E, Emshoff A, Ito A, Robberts JM, Delker C, 2003. Echinococcosis on the Tibetan Plateau: prevalence and risk factors for cystic and alveolar echinococcosis in Tibetan populations in Qinghai Province, China. *Parasitology* 127: S109–S120.
11. Nakao M, Sako Y, Ito A, 2003. Isolation of polymorphic microsatellite loci from the tapeworm *Echinococcus multilocularis*. *Infect Genet Evol* 3: 159–163.
12. Štefanić S, Shaikenov BS, Deplazes P, Dinkel A, Torgerson PR, Mathis A, 2004. Polymerase chain reaction for detection of patent infections of *Echinococcus granulosus* (“sheep strain”) in naturally infected dogs. *Parasitol Res* 92: 347–351.
13. Thomson RCA, Eckert J, 1983. Observations on *Echinococcus multilocularis* in the definitive host. *Z Parasitenkd* 69: 335–345.
14. Zhang LH, Chai JJ, Jiao W, Osman Y, McManus DP, 1998. Mitochondrial genomic markers confirm the presence of the camel strain (G6 genotype) of *Echinococcus granulosus* in north-western China. *Parasitology* 116: 29–33.