Appendix A. List of experts

Argentina	Bernardo Frider	Division of Internal Medicine-Hepatology; Argerich Hospital, Government of Buenos Aires City, Associated to the University of Buenos Aires, 1171 Buenos Aires, Argentina	frider@bigfoot.com
Bulgaria	Kirien Kjossev	Department of General Surgery; Military Medical Academy, Sofia, Bulgaria	kirien@abv.bg
	Vladimir Prandjev	Department of Neurosurgery; Military Medical Academy, Sofia, Bulgaria	prandjev@hotmail.com
France	Karine Bardonnet	CCOMS; Dept of Biochemistry; University Hospital, 25030 Besançon, France	kbardonnet@chu-besancon.fr
	Brigitte Bartholomot	CCOMS; Centre de Radiologie des Deux-Princesses, 25000 Besançon, France	brigitte.bartholomot@wanadoo.fr
	Oleg Blagosklonov	CCOMS; Dept of Nuclear Medicine; University Hospital, 25030 Besançon, France	oleg.blagosklonov@univ-fcomte.fr
	Franck Boué	Agence Française de Sécurité Sanitaire des Aliments ; Technopôle Agricole et Vétérinaire, 54220 Malzéville, France	f.boue@afssa.fr
	Solange Bresson-Hadni	CCOMS; Dept of Digestive and Cancer Surgery; University Hospital, 25030 Besançon, France	dr.bresson.hadni@wanadoo.fr; ccoms@chu-besancon.fr
	Eric Delabrousse	CCOMS; Dept of Radiology A; University Hospital, 25030 Besançon, France	e.delabrousse@chu-besancon.fr
	Alain Gérard	Dept of Infectious Diseases; University Hospital Brabois, 54500 Vandoeuvre les Nancy, France	a.gerard@chu-nancy.fr
	Patrick Giraudoux	CCOMS; UMR Chrono-Environnement; Université de Franche-Comté, 25030 Besançon	patrick.giraudoux@univ-fcomte.fr
	Jenny Knapp	CCOMS; UMR Chrono-Environnement; Université de Franche-Comté, 25030 Besançon	patrick.giraudoux@univ-fcomte.fr
	Georges Mantion	CCOMS; Dept of Digestive and Cancer Surgery; University Hospital, 25030 Besançon, France	gmantion@univ-fcomte.fr
	Laurence Millon	CCOMS; Dept of Parasitology; University Hospital, and UMR Chrono-Environnement; Université de Franche-Comté, 25030 Besançon	laurence.millon@univ-fcomte.fr
	Martine Piarroux	CCOMS; FrancEchino Network; University Hospital, 25030 Besançon	mpiarroux@yahoo.fr
	Renaud Piarroux	Dept of Parasitology; University Hospital La Timone, 13385 Marseille cedex 5, France	Renaud.Piarroux@mail.ap-hm.fr
	Francis Raoul	CCOMS; UMR Chrono-Environnement; Université de Franche-Comté, 25030 Besançon	francis.raoul@univ-fcomte.fr
	Anne-Laure Rayssac and Carine Richou	CCOMS; Dept of Hepatology; University Hospital, 25030 Besançon	crichou@chu-besancon.fr
	Dominique A Vuitton	CCOMS; EA 3181; Université de Franche-Comté, 25030 Besançon	dominique.vuitton@univ-fcomte.fr
	Jérôme Watelet	Dept of Gastroenterology; University Hospital Brabois, 54500 Vandoeuvre les Nancy, France	j.watelet@chu-nancy.fr
Germany	Holger Barth	Dept of Surgery; University Hospital, 89081 Ulm, Germany	Holger.Barth@uni-ulm.de
	Klaus Buttenschön	Dept of Surgery; University Hospital, 89081 Ulm, Germany	klaus.buttenschoen@uniklinik-ulm.de
	Waldemar Hosch	Dept of Radiology; University Hospital; 69120 Heidelberg, Germany	waldemar.hosch@urz.uni-heidelberg.de
	Thomas Junghanss	Section of Clinical Tropical Medicine; University Hospital; 69120 Heidelberg	Thomas.Junghanss@urz.uni-heidelberg.de
	Peter Kern	Sektion Infektiologie und Klinische Immunologie; University Hospital, 89081 Ulm, Germany	peter.kern@uniklinik-ulm.de
	Stefan Reuter	Sektion Infektiologie und Klinische Immunologie, University Hospital, 89081 Ulm, Germany	stefan.reuter@uniklinik-ulm.de
	Hanns M Seitz	Henri-Spaakstr. 161, 53123 Bonn, Germany	seitz@parasit.meb.uni-bonn.de
Iraq	Nadham Mahdi	College of Medicine; University of Basrah, 1565 BASRAH, Iraq	nadhammahdi@yahoo.com
Italy	Giorgio Battelli	Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale; Facoltà di Medicina veterinaria, 40064 Ozzano Emilia (Bologna), Italia	giorgio.battelli@unibo.it
	Enrico Brunetti	Division of Infectious and Tropical Diseases; IRCCS S. Mateo, Pavia University, Pavia, Italy	selim@unipv.it
	Carlo Filice	Division of Infectious and Tropical Diseases; IRCCS S. Mateo, Pavia University, Pavia, Italy	carfil@unipv.it
	Antonella Teggi	Dipartimento di Malattie Infettive; Ospedale Sant'Andrea, University "La Sapienza", 00189 Rome, Italy	spec.trop@tiscali.it
Japan	Akira Ito	Dept of Parasitology; Asahikawa University, 078-8510 Asahikawa, Japan	akiraito@asahikawa-med.ac.jp
	Naoki Sato	Dept of Digestive Surgery; University Hospital, 060-8638 Sapporo, Japan	naoki-sa@med.hokudai.ac.jp
Marocco	Mustapha Benazzouz	Unité d'Hépato-Gastro-Entérologie, Médecine C, Hopital Ibn Sina, BP1005 Rabat, Morocco	benazmusta@menara.ma
Netherlands	Hans G Schipper	Department of Infectious Diseases, Tropical Medicine, and AIDS; Academic Medical Centre, 1105 AZ, Amsterdam,The Netherlands	h.g.schipper@amc.uva.nl
PR China	Fang Ping He	Dept of Hepatology; Key Lab for basic and clinical research on echinococcosis; Xinjiang Medical University 1st Teaching Hospital, 830054 Urumqi, China	hefp2009@163.com
	Wen Ya Liu	Dept of Radiology; Xinjiang Medical University 1st Teaching Hospital, 830054 Urumqi, China	wenyaliu2002@yahoo.com.cn
	Xiao Mei Lu	Dept of Clinical research; Key Lab for basic and clinical research on echinococcosis; Xinjiang Medical University 1st Teaching Hospital, 830054 Urumqi, China	luxiaomei88@163.com
	Shu Mei Ma	Dept of Parasitology; Qinghai Medical College, Xinning, Qinghai,	

Appendix A (*Continued*)

PR China	mashumei411@sohu.com		
	XinYu Peng	Dept of Surgery; Shihezi University Medical College; 832008 Shihezi, Xinjiang, China	pengxy2000@yahoo.com
	Ying Mei Shao	Dept of Digestive and Hepatic Surgery; Key Lab for basic and clinical research on echinococcosis; Xinjiang Medical University 1st Teaching Hospital, 830054 Urumqi, China	shaoym@lzu.edu.cn
	Jian Hua Wang	Dept of Pharmacology; Xinjiang Medical University, 830054 Urumqi, China	jhw716@163.com
	Yun Hai Wang	Dept of Digestive and Hepatic Surgery; Key Lab for basic and clinical research on echinococcosis; 1st Teaching Hospital of Xinjiang Medical University; Urumqi, China	yunhai_wang@hotmail.com
	Hao Wen	Dept of Digestive and Hepatic Surgery; Key Lab for basic and clinical research on echinococcosis; 1st Teaching Hospital of Xinjiang Medical University; Urumqi, China	dr.wenhao@163.com
	Yu Rong Yang	Molecular Parasitology Laboratory; Queensland Institute of Medical Research, Brisbane, Q4006, Australia (and Ningxia Medical College, Yinshuan, China)	yurongY@qimr.edu.au
Perù	Hector H. Garcia	Laboratorio de Parasitologia; Universidad Peruana Cayetano Heredia, SMP, Lima, Peru	hgarcia@jhsph.edu
Portugal	Antonio Menezes da Silva	Dept of Surgery, Hospital Pulido Valente Alameda das Linhas de Torres, 1600 Lisbon, Portugal	mensilvapt@yahoo.com
Romania	Carmen Cretu	Parasitology Department; "Carol Davila" University of Medicine and Pharmacy, 020027, Bucharest, Romania	michaelacarmen.cretu@gmail.com
Serbia	Miroslav Milicevic	Institute for Digestive Diseases; First Surgical Clinic University of Belgrade Clinical Centre, 11000 Belgrade, Serbia	machak@sbb.co.yu
Spain	Rogelio Lopez-Velez	Medicina Tropical y Parasitología Clínica; Servicio de Enfermedades Infecciosas; Hospital Ramón y Cajal. 28034 Madrid, Spain	rlopezvelez.hrc@salud.madrid.org
Switzerland	Bruno Gottstein	Parasitology Institute; Bern University, 3012 Bern, Switzerland	bruno.gottstein@ipa.unibe.ch
	Beat Müllhaupt	Departement für Innere Medizin, Gastroenterologie; Universitätsspital, 8091 Zürich, Switzerland	beat.muellhaupt@usz.ch
Tunisia	Chadli Dziri	Division of General Surgery, Department of Emergency; Hôpital Charles Nicolle, 1006 Tunis, Tunisia	chadli.dziri@planet.tn
Turkey	Okan Akhan	Radyoloji AD, Hacettepe Universitesi, 06100 Ankara, Turkey	akhano@tr.net
	Erbug Keskin	Dept of Paediatric Surgery; University of Cukurova, 01330 Adana, Turkey	erbug@erbug.net
West Indies	Calum Macpherson	Dept of parasitology; St George's University St George's, Grenada, WI	cmacpherson@sgu.edu
USA	Peter M.S. Schantz	Division of Parasitic Diseases; National Center For Infectious Diseases, Centers For Disease Control and Prevention, Atlanta, GA 30341, USA	p.schantz@comcast.net
UK	Peter Chiodini	Department of Clinical Parasitology; Hospital for Tropical Diseases, London WC1E 6JB, UK	peter.chiodini@uclh.nhs.uk
	Philip S. Craig	Biosciences Research Institute; School of Environment & Life Sciences; University of Salford, Salford M5 4WT, UK	p.s.craig@salford.ac.uk
	John Horton	24 The Paddock, Hitchin, Herts. SG4 9EF, UK	Phone: +44-1462-624081; mobile: +44-7881815363 hedgepigs@aol.com
	Michael Rogan	Biosciences Research Institute; School of Environment & Life Sciences; University of Salford, Salford M5 4WT, UK	M.T.Rogan@salford.ac.uk
UN/WHO	Albis Gabrielli	NTD Department, World Health Organisation, 1211 Genève	gabrielli@who.int
	François-Xavier Meslin	NTD Department, World Health Organisation, 1211 Genève	meslin@who.int

References

Akhan, O., Gumus, B., Akinci, D., Karcaaltincaba, M., Ozmen, M., 2007. Diagnosis and percutaneous treatment of soft-tissue hydatid cysts. Cardiovasc. Intervent. Radiol. 30, 419–425.

Akisu, C., Delibas, S.B., Bicmen, C., Ozkoc, S., Aksoy, U., Turgay, N., 2006. Comparative evaluation of western blotting in hepatic and pulmonary cystic echinococcosis. Parasite 13, 321–326.

Ammann, R.W., Eckert, J., 1996. Cestodes. *Echinococcus*. Gastroenterol. Clin. North Am. 25, 655–689.

Ammann, R.W., Renner, E.C., Gottstein, B., Grimm, F., Eckert, J., Renner, E.L., 2004. Immunosurveillance of alveolar echinococcosis by specific humoral and cellular immune tests: long-term analysis of the Swiss chemotherapy trial (1976–2001). J. Hepatol. 41, 551–559.

Arif, S.H., Shams Ul, B., Wani, N.A., Zargar, S.A., Wani, M.A., Tabassum, R., Hussain, Z., Baba, A.A., Lone, R.A., 2008. Albendazole as an adjuvant to the standard surgical management of hydatid cyst liver. Int. J. Surg. 6, 448–451.

Aydin, U., Yazici, P., Onen, Z., Ozsoy, M., Zeytunlu, M., Kilic, M., Coker, A., 2008. The optimal treatment of hydatid cyst of the liver: radical surgery with a significant reduced risk of recurrence. Turk. J. Gastroenterol. 19, 33–39.

Bartholomot, G., Vuitton, D.A., Harraga, S., Shida, Z., Giraudoux, P., Barnish, G., Wang, Y.H., MacPherson, C.N., Craig, P.S., 2002. Combined ultrasound and serologic screening for hepatic alveolar echinococcosis in central China. Am. J. Trop. Med. Hyg. 66, 23–29.

Baskaran, V., Patnaik, P.K., 2004. Feasibility and safety of laparoscopic management of hydatid disease of the liver. JSLS 8, 359–363.

Ben Nouir, N., Nunez, S., Gianinazzi, C., Gorcii, M., Muller, N., Nouri, A., Babba, H., Gottstein, B., 2008. Assessment of *Echinococcus granulosus* somatic protoscolex antigens for serological follow-up of young patients surgically treated for cystic echinococcosis. J. Clin. Microbiol. 46, 1631–1640.

Bradley, M., Horton, J., 2001. Assessing the risk of benzimidazole therapy during pregnancy. Trans. R. Soc. Trop. Med. Hyg. 95, 72–73.

Bresson-Hadni, S., Delabrousse, E., Blagosklonov, O., Bartholomot, B., Koch, S., Miguet, J.P., Mantion, G., Vuitton, D.A., 2006. Imaging aspects and non-surgical interventional treatment in human alveolar echinococcosis. Parasitol. Int. 55 (Suppl.), S267–S272.

Bresson-Hadni, S., Koch, S., Miguet, J.P., Gillet, M., Mantion, G.A., Heyd, B., Vuitton, D.A., 2003. Indications and results of liver transplantation for *Echinococcus* alveolar infection: an overview. Langenbecks Arch. Surg. 388, 231–238.

Bresson-Hadni, S., Laplante, J.J., Lenys, D., Rohmer, P., Gottstein, B., Jacquier, P., Mercet, P., Meyer, J.P., Miguet, J.P., Vuitton, D.A., 1994. Seroepidemiologic screening of *Echinococcus multilocularis* infection in a European area endemic for alveolar echinococcosis. Am. J. Trop. Med. Hyg. 51, 837–846.

Bresson-Hadni, S., Vuitton, D.A., Bartholomot, B., Heyd, B., Godart, D., Meyer, J.P., Hrusovsky, S., Becker, M.C., Mantion, G., Lenys, D., Miguet, J.P., 2000. A twenty-year history of alveolar echinococcosis: analysis of a series of 117 patients from eastern France. Eur. J. Gastroenterol. Hepatol. 12, 327–336.

Budke, C.M., 2006. Global socioeconomic impact of cystic echinococcosis. Emerg. Infect. Dis. 12, 296–303.

Buttenschoen, K., Carli Buttenschoen, D., Gruener, B., Kern, P., Beger, H.G., Henne-Bruns, D., Reuter, S., 2009. Long-term experience on surgical treatment of alveolar echinococcosis. Langenbecks Arch. Surg. 394, 698.

Buttenschoen, K., Carli Buttenschoen, D., Gruener, B., Kern, P., Beger, H.G., Henne-Bruns, D., Reuter, S., 2009a. Long-term experience on surgical treatment of alveolar echinococcosis. Langenbecks Arch. Surg. 394, 689–698.

Buttenschoen, K., Gruener, B., Carli Buttenschoen, D., Reuter, S., Henne-Bruns, D., Kern, P., 2009b. Palliative operation for the treatment of alveolar echinococcosis. Langenbecks Arch. Surg. 394, 199–204.

Bygott, J.M., Chiodini, P.L., 2009. Praziquantel: neglected drug? Ineffective treatment? Or therapeutic choice in cystic hydatid disease? Acta Trop. 111, 95–101.

Chawla, A., Maheshwari, M., Parmar, H., Hira, P., Hanchate, V., 2003. Imaging features of disseminated peritoneal hydatidosis before and after medical treatment. Clin. Radiol. 58, 818–820.
Cobo, F., Yarnoz, C., Sesma, B., Fraile, P., Aizcorbe, M., Trujillo, R., Diaz-de-Liano, A., Ciga, M.A., 1998. Albendazole plus praziquantel versus albendazole alone as a pre-operative treatment in intra-abdominal hydatisosis caused by *Echinococcus granulosus*. Trop. Med. Int. Health 3, 462–466.
Craig, P., 2003. *Echinococcus multilocularis*. Curr. Opin. Infect. Dis. 16, 437–444.
Craig, P., Budke, C.M., Schantz, P.M., Tiaoying, L., Qiu, J.Y.Y., Zehyle, E., Rogan, M.T., Ito, A., 2007. Human echinococcosis: a neglected disease? Trop. Med. Health 35, 283–292.
Dogru, D., Kiper, N., Ozcelik, U., Yalcin, E., Gocmen, A., 2005. Medical treatment of pulmonary hydatid disease: for which child? Parasitol. Int. 54, 135–138.
Dziri, C., Haouet, K., Fingerhut, A., 2004. Treatment of hydatid cyst of the liver: where is the evidence? World J. Surg. 28, 731–736.
Dziri, C., Haouet, K., Fingerhut, A., Zaouche, A., 2009. Management of cystic echinococcosis complications and dissemination: where is the evidence? World J. Surg. 33, 1266–1273.
El Fortia, M., El Gatit, A., Bendaoud, M., 2006. Ultrasound wall-sign in pulmonary echinococcosis (new application). Ultraschall Med. 27, 553–557.
Franchi, C., Di Vico, B., Teggi, A., 1999. Long-term evaluation of patients with hydatidosis treated with benzimidazole carbamates. Clin. Infect. Dis. 29, 304–309.
Frider, B., Larrieu, E., Odriozola, M., 1999. Long-term outcome of asymptomatic liver hydatidosis. J. Hepatol. 30, 228–231.
Galitza, Z., Bazarsky, E., Sneier, R., Peiser, J., El-On, J., 2006. Repeated treatment of cystic echinococcosis in patients with a long-term immunological response after successful surgical cyst removal. Trans. R. Soc. Trop. Med. Hyg. 100, 126–133.
Gharbi, H.A., Hassine, W., Brauner, M.W., Dupuch, K., 1981. Ultrasound examination of the hydatic liver. Radiology 139, 459–463.
Gottstein, B., Saucy, F., Deplazes, P., Reichen, J., Demierre, G., Busato, A., Zuercher, C., Pugin, P., 2001. Is high prevalence of *Echinococcus multilocularis* in wild and domestic animals associated with disease incidence in humans? Emerg. Infect. Dis. 7, 408–412.
Haddad, M.C., Sammak, B.M., Al-Karawi, M., 2000. Percutaneous treatment of heterogenous predominantly solid echopattern echinococcal cysts of the liver. Cardiovasc. Intervent. Radiol. 23, 121–125.
Hosch, W., Junghanss, T., Stojkovic, M., Brunetti, E., Heye, T., Kauffmann, G.W., Hull, W.E., 2008. Metabolic viability assessment of cystic echinococcosis using high-field 1H MRS of cyst contents. NMR Biomed. 21, 734–754.
Hosch, W., Stojkovic, M., Janisch, T., Kauffmann, G.W., Junghanss, T., 2007. The role of calcification for staging cystic echinococcosis (CE). Eur. Radiol. 17, 2538–2545.
Isitmangil, T., Sebit, S., Tunc, H., Gorur, R., Erdik, O., Kunter, E., Toker, A., Balkanli, K., Ozturk, O.Y., 2002. Clinical experience of surgical therapy in 207 patients with thoracic hydatidosis over a 12-year-period. Swiss Med. Wkly. 132, 548–552.
Ito, A., Craig, P.S., 2003. Immunodiagnostic and molecular approaches for the detection of taeniid cestode infections. Trends Parasitol. 19, 377–381.
Junghanss, T., Menezes da Silva, A., Horton, J., Chiodini, P.L., Brunetti, E., 2008. Clinical management of cystic echinococcosis: state of the art, problems, and perspectives. Am. J. Trop. Med. Hyg. 79, 301–311.
Kadry, Z., Renner, E.C., Bachmann, L.M., Attigah, N., Renner, E.L., Ammann, R.W., Clavien, P.A., 2005. Evaluation of treatment and long-term follow-up in patients with hepatic alveolar echinococcosis. Br. J. Surg. 92, 1110–1116.
Kern, P., Abboud, P., Kern, W., Stich, A., Bresson-Hadni, S., Guerin, B., Buttenschoen, K., Gruener, B., Reuter, S., Hemphill, A., 2008. Critical appraisal of nitazoxanide for the treatment of alveolar echinococcosis. Am. J. Trop. Med. Hyg. 79, 119.
Kern, P., Bardonnet, K., Renner, E., Auer, H., Pawlowski, Z., Ammann, R.W., Vuitton, D.A., 2003. European echinococcosis registry: human alveolar echinococcosis, Europe, 1982–2000. Emerg. Infect. Dis. 9, 343–349.
Kern, P., Wen, H., Sato, N., Vuitton, D.A., Gruener, B., Shao, Y., Delabrousse, E., Kratzer, W., Bresson-Hadni, S., 2006. WHO classification of alveolar echinococcosis: principles and application. Parasitol. Int. 55 (Suppl.), S283–S287.
Khabiri, A.R., Bagheri, F., Assmar, M., Siavashi, M.R., 2006. Analysis of specific IgE and IgG subclass antibodies for diagnosis of *Echinococcus granulosus*. Parasite Immunol. 28, 357–362.
Khuroo, M.S., Dar, M.Y., Yattoo, G.N., Zargar, S.A., Javaid, G., Khan, B.A., Boda, M.I., 1993. Percutaneous drainage versus albendazole therapy in hepatic hydatidosis: a prospective, randomized study. Gastroenterology 104, 1452–1459.
Kish, M.A., 2001. Guide to development of practice guidelines. Clin. Infect. Dis. 32, 851–854.
Kjossev, K.T., Losanoff, J.E., 2005. Classification of hydatid liver cysts. J. Gastroenterol. Hepatol. 20, 352–359.
Koch, S., Bresson-Hadni, S., Miguet, J.P., Crumbach, J.P., Gillet, M., Mantion, G.A., Heyd, B., Vuitton, D.A., Minello, A., Kurtz, S., 2003. Experience of liver transplantation for incurable alveolar echinococcosis: a 45-case European collaborative report. Transplantation 75, 856–863.
Larrieu, E.J., Frider, B., 2001. Human cystic echinococcosis: contributions to the natural history of the disease. Ann. Trop. Med. Parasitol. 95, 679–687.
Macpherson, C.N., Milner, R., 2003. Performance characteristics and quality control of community based ultrasound surveys for cystic and alveolar echinococcosis. Acta Trop. 85, 203–209.
Marchiondo, A.A., Ming, R., Andersen, F.L., Slusser, J.H., Conder, G.A., 1994. Enhanced larval cyst growth of *Echinococcus multilocularis* in praziquantel-treated jirds (*Meriones unguiculatus*). Am. J. Trop. Med. Hyg. 50, 120–127.
McManus, D.P., Zhang, W., Li, J., Bartley, P.B., 2003. Echinococcosis. Lancet 362, 1295–1304.
Men, S., Yucesoy, C., Edguer, T.R., Hekimoglu, B., 2006. Percutaneous treatment of giant abdominal hydatid cysts: long-term results. Surg. Endosc. 20, 1600–1606.
Menezes da Silva, A., 2003. Hydatid cyst of the liver-criteria for the selection of appropriate treatment. Acta Trop. 85, 237–242.
Morris, D.L., Taylor, D.H., 1988. Optimal timing of post-operative albendazole prophylaxis in *E. granulosus*. Ann. Trop. Med. Parasitol. 82, 65–66.
Mufit, K., Nejat, I., Mercan, S., Ibrahim, K., Mete, U.Y., Yuksel, K., 1998. Growth of multiple hydatid cysts evaluated by computed tomography. J. Clin. Neurosci. 5, 215–217.
Peng, X., Zhang, S., Niu, J.H., 2002. Total subadventitial cystectomy for the treatment of 30 patients with hepatic hydatid cysts. Chin. J. Gen. Surg. 17, 529–530.
Rausch, R.L., Wilson, J.F., Schantz, P.M., McMahon, B.J., 1987. Spontaneous death of *Echinococcus multilocularis*: cases diagnosed serologically (by Em2 ELISA) and clinical significance. Am. J. Trop. Med. Hyg. 36, 576–585.
Reuter, S., Buck, A., Grebe, O., Nussle-Kugele, K., Kern, P., Manfras, B.J., 2003. Salvage treatment with amphotericin B in progressive human alveolar echinococcosis. Antimicrob. Agents Chemother. 47, 3586–3591.
Reuter, S., Buck, A., Manfras, B., Kratzer, W., Seitz, H.M., Darge, K., Reske, S.N., Kern, P., 2004. Structured treatment interruption in patients with alveolar echinococcosis. Hepatology 39, 509–517.
Reuter, S., Jensen, B., Buttenschoen, K., Kratzer, W., Kern, P., 2000. Benzimidazoles in the treatment of alveolar echinococcosis: a comparative study and review of the literature. J. Antimicrob. Chemother. 46, 451–456.
Rogan, M.T., Hai, W.Y., Richardson, R., Zeyhle, E., Craig, P.S., 2006. Hydatid cysts: does every picture tell a story? Trends Parasitol. 22, 431–438.
Romig, T., Kratzer, W., Kimmig, P., Frosch, M., Gaus, W., Flegel, W.A., Gottstein, B., Lucius, R., Beckh, K., Kern, P., 1999. An epidemiologic survey of human alveolar echinococcosis in southwestern Germany. Romerstein Study Group. Am. J. Trop. Med. Hyg. 61, 566–573.
Romig, T., Zeyhle, E., Macpherson, C.N., Rees, P.H., Were, J.B., 1986. Cyst growth and spontaneous cure in hydatid disease. Lancet 1, 861.
Saremi, F., McNamara, T.O., 1995. Hydatid cysts of the liver: long-term results of percutaneous treatment using a cutting instrument. Am. J. Roentgenol. 165, 1163–1167.
Sato, N., Namieno, T., Furuya, K., Takahashi, H., Yamashita, K., Uchino, J., Suzuki, K., 1997. Contribution of mass screening system to resectability of hepatic lesions involving *Echinococcus multilocularis*. J. Gastroenterol. 32, 351–354.
Scheuring, U.J., Seitz, H.M., Wellmann, A., Hartlapp, J.H., Tappe, D., Brehm, K., Spengler, U., Sauerbruch, T., Rockstroh, J.K., 2003. Long-term benzimidazole treatment of alveolar echinococcosis with hematogenic subcutaneous and bone dissemination. Med. Microbiol. Immunol. (Berl.) 192, 193–195.
Schipper, H.G., Lameris, J.S., van Delden, O.M., Rauws, E.A., Kager, P.A., 2002. Percutaneous evacuation (PEVAC) of multivesicular echinococcal cysts with or without cystobiliary fistulas which contain non-drainable material: first results of a modified PAIR method. Gut 50, 718–723.
Schweiger, A., Ammann, R.W., Candinas, D., Clavien, P.A., Eckert, J., Gottstein, B., Halkic, N., Muellhaupt, B., Prinz, B.M., Reichen, J., Tarr, P.E., Torgerson, P.R., Deplazes, P., 2007. Human alveolar echinococcosis after fox population increase, Switzerland. Emerg. Infect. Dis. 13, 878–882.
Seckin, H., Yagmurlu, B., Yigitkanli, K., Kars, H.Z., 2008. Metabolic changes during successful medical therapy for brain hydatid cyst: case report. Surg. Neurol. 70, 186–189.
Siles-Lucas, M.M., Gottstein, B.B., 2001. Molecular tools for the diagnosis of cystic and alveolar echinococcosis. Trop. Med. Int. Health 6, 463–475.
Siracusano, A., Bruschi, F., 2006. Cystic echinococcosis: progress and limits in epidemiology and immunodiagnosis. Parassitologia 48, 65–66.
Smego Jr., R.A., Bhatti, S., Khaliq, A.A., Beg, M.A., 2003. Percutaneous aspiration-injection-reaspiration drainage plus albendazole or mebendazole for hepatic cystic echinococcosis: a meta-analysis. Clin. Infect. Dis. 37, 1073–1083.
Stojkovic, M., Zwahlen, M., Teggi, A., Vutova, K., Cretu, C.M., Virdone, R., Nicolaidou, P., Cobanoglu, N., Junghanss, T., 2009. Treatment response of cystic echinococcosis to benzimidazoles: a systematic review. PLoS Negl. Trop. Dis. 3, e524.
Stumpe, K.D., Renner-Schneiter, E.C., Kuenzle, A.K., Grimm, F., Kadry, Z., Clavien, P.A., Deplazes, P., von Schulthess, G.K., Muellhaupt, B., Ammann, R.W., Renner, E.L., 2007. F-18-fluorodeoxyglucose (FDG) positron-emission tomography of *Echinococcus multilocularis* liver lesions: prospective evaluation of its value for diagnosis and follow-up during benzimidazole therapy. Infection 35, 11–18.
Tappe, D., Frosch, M., Sako, Y., Itoh, S., Gruener, B., Reuter, S., Nakao, M., Ito, A., Kern, P., 2009. Close relationship between clinical regression and specific serology in the follow-up of patients with alveolar echinococcosis in different clinical stages. Am. J. Trop. Med. Hyg. 80, 792–797.
Thameur, H., Abdelmoula, S., Chenik, S., Bey, M., Ziadi, M., Mestiri, T., Mechmeche, R., Chaouch, H., 2001. Cardiopericardial hydatid cysts. World J. Surg. 25, 58–67.
Torgerson, P.R., Schweiger, A., Deplazes, P., Pohar, M., Reichen, J., Ammann, R.W., Tarr, P.E., Halkik, N., Mullhaupt, B., 2008. Alveolar echinococcosis: from a deadly disease to a well-controlled infection. Relative survival and economic analysis in Switzerland over the last 35 years. J. Hepatol. 49, 72–77.
Uchino, J., Sato, N., Nakajima, Y., Matsushita, M., Takahashi, M., Une, Y., 1993. Treatment. In: Uchino, J., Sato, N. (Eds.), Alveolar Echinococcosis of the Liver. Hokkaido University School of Medicine, Sapporo.
Ustunsoz, B., Akhan, O., Kamiloglu, M.A., Somuncu, I., Ugurel, M.S., Cetiner, S., 1999. Percutaneous treatment of hydatid cysts of the liver: long-term results. Am. J. Roentgenol. 172, 91–96.
Ustunsoz, B., Ugurel, M.S., Uzar, A.I., Duru, N.K., 2008. Percutaneous treatment of hepatic hydatid cyst in pregnancy: long-term results. Arch. Gynecol. Obstet. 277, 547–550.

Vuitton, D., Zhang, S.L., Yang, Y., Godot, V., Beurton, I., Mantion, G., Bresson-Hadni, S., 2006. Survival strategy of *Echinococcus multilocularis* in the human host. Parasitol. Int. 55(Suppl.), S51–S55.

Vuitton, D.A., Zhi Wang, X., Li Feng, S., Cheng Shen, J., Shou Li, Y., Li, S.F., Ke Tang, Q., 2002. PAIR-derived US-guided techniques for the treatment of cystic echinococcosis: a Chinese experience (e-letter). Gut.

Vutova, K., Mechkov, G., Vachkov, P., Petkov, R., Georgiev, P., Handjiev, S., Ivanov, A., Todorov, T., 1999. Effect of mebendazole on human cystic echinococcosis: the role of dosage and treatment duration. Ann. Trop. Med. Parasitol. 93, 357–365.

Wang, X., Li, Y., Feng, S., 1994. [Clinical treatment of hepatic and abdominal hydatid cyst by percutaneous puncture, drainage and curettage] Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 12, 285–287.

Wang, Y., He, T., Wen, X., Li, T., Waili, A., Zhang, W., Xu, X., Vuitton, D.A., Rogan, M.T., Wen, H., Craig, P.S., 2006. Post-survey follow-up for human cystic echinococcosis in northwest China. Acta Trop. 98, 43–51.

WHO-Informal Working Group on Echinococcosis, 1996. Guidelines for treatment of cystic and alveolar echinococcosis in humans. Bull. WHO 74, 231–242.

WHO/OIE Manual on Echinococcosis, 2001. Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern. World Organisation for Animal Health (Office International des Epizooties) and World Health Organisation.

WHO-Informal Working Group on Echinococcosis, 2003a. PAIR: Puncture, Aspiration, Injection, Re-Aspiration. An Option for the Treatment of Cystic Echinococcosis. WHO, Geneva.

WHO-Informal Working Group on Echinococcosis, 2003b. International classification of ultrasound images in cystic echinococcosis for application in clinical and field epidemiological settings. Acta Trop. 85, 253–261.

Yamasaki, H., Nakaya, K., Nakao, M., Sako, Y.A.I., 2007. Significance of molecular diagnosis using histopathological specimens in cestode zoonoses. Trop. Med. Health 35, 307–321.

Zlitni, M., Ezzaouia, K., Lebib, H., Karray, M., Kooli, M., Mestiri, M., 2001. Hydatid cyst of bone: diagnosis and treatment. World J. Surg. 25, 75–82.

Journal of TRAVEL MEDICINE

Neurocysticercosis: Assessing Where the Infection Was Acquired From

Tetsuya Yanagida, PhD,* Izumi Yuzawa, MD,† Durga D. Joshi, PhD,‡ Yasuhito Sako, PhD,* Minoru Nakao, PhD,* Kazuhiro Nakaya, PhD,§ Nobuyuki Kawano, MD,‖ Hidehiro Oka, MD,† Kiyotaka Fujii, MD,† and Akira Ito, PhD*

*Department of Parasitology, Asahikawa Medical College, Hokkaido, Japan; †Department of Neurosurgery, School of Medicine, Kitasato University, Kanagawa, Japan; ‡National Zoonoses and Food-Hygiene Research Center, Kathmandu, Nepal; §Animal Laboratory for Medical Research, Asahikawa Medical College, Hokkaido, Japan; ‖Isobe Clinic, Kanagawa, Japan

DOI: 10.1111/j.1708-8305.2010.00409.x

Histopathological specimen of a neurocysticercosis patient, who had been living in several endemic countries, was retrospectively analyzed for assessing the origin of the infection. Mitochondrial DNA analysis strongly suggested that the patient became infected with the parasite in Nepal at least 10 years before the onset of the disease.

The pork tapeworm *Taenia solium* is one of the most important human parasites because of its pathogenicity. It causes two types of human infection: (1) teniasis, intestinal infection of adult worms, caused by eating undercooked pork contaminated with cysticerci (larval stage) and (2) cysticercosis, tissue infection of cysticerci throughout the body, acquired by ingesting the eggs. Neurocysticercosis (NCC), cysticercosis in the central nervous system, is a lethal and rather common parasitic disease in many developing countries where pork is consumed. However, the recent increase in the number of immigrants and tourists is spreading the disease into the more developed countries and the communities where eating pork is forbidden.[1–3] Thus, it is important to ascertain the origin of the infection to assess the risk factors in nonendemic areas.[4]

The Study

In August 1996, a 46-year-old Japanese man complained of a dull headache during his stay in Jakarta, Indonesia, and he had a medical examination at the local hospital in Manila, Philippines on the way back to Japan. Cerebral computer tomography (CT) showed a putative brain tumor in the left temporal lobe. Then he came back to Japan and was admitted to Kitasato University Hospital. By CT scanning, a small solitary cystic lesion with ring enhancement was observed at the cerebral surface of the left temporal lobe. He showed no other neurological abnormalities, and his blood/stool tests were within the normal range. In September, the patient was operated and a well-encapsulated cyst of about 1 cm in diameter was surgically resected. Histopathological examination revealed it to be a viable cysticercus of *T. solium*.[5] He recovered well and came back to his job 19 days after the operation. NCC with solitary cyst was later confirmed serologically using highly specific antigens at Asahikawa Medical College.[6]

Where did the patient become infected? Because teniasis/cysticercosis is not endemic in Japan, it was assumed that he acquired the parasite outside of Japan. He had been an overseas technical advisor for 12 years since 1970s, and visited Indonesia, Nigeria, Nepal, and Malaysia, where NCC cases have been reported.[7] Because the patient had lived in Indonesia for 6 years just before the onset of the disease (1990–1996), we suspected that he had been infected with the parasite there. To solve the puzzle, we retrospectively analyzed cytochrome *c* oxidase I (*cox1*) of mitochondrial DNA (mtDNA) using the formalin-fixed and paraffin-embedded histological specimen prepared from the patient. Based on the phylogenetic analysis using

Corresponding Author: Tetsuya Yanagida, PhD, Department of Parasitology, Asahikawa Medical College, Hokkaido 078-8510, Japan. E-mail: yanagida@asahikawa-med.ac.jp

Table 1 Primers used for the amplification of *cox1* gene fragments of *Taenia solium*

Primers	Sequence (5′ to 3′)	Positions	Reference
Tsol cox1/F00	atggttttattagtgcgtc	1–19	PS
Tsol cox1/F100	taagttttagtttattaattcgtgt	101–125	PS
Tsol cox1/F180	gattactaaccatggtataataatg	179–204	PS
Tsol cox1/F300	ctttaagtgcatggttgtt	305–323	PS
Tsol cox1/F380	ggacttttacccaccttttatcatct	379–405	PS
Tsol cox1/F500	tatgtacattatatagagtttttatgact	497–525	PS
Taenia cox F3	tatttgatcgtaaatttagttctgcgtt	629–656	9
Tsol cox1/F850	tttaggaagaagtgtgtgagg	846–866	PS
Tsol cox1/F950	gattaaggtttttacttggc	951–970	PS
Tsol cox1/F1080	tctgcttgtgtattagataaagt	1078–1100	PS
Tsol cox1/F1170	ttatgtttgtttggtggtga	1175–1194	PS
Tsol cox1/F1290	ttttgggttgtgtgggtta	1293–1311	PS
Tsol cox1/F1420	gagagtcagttgttaatcgtaaa	1421–1443	PS
Tsol cox1/R1620	ctaaaagaccattccacacgcgaatac	1620–1594	PS
Taenia cox R6	acaggactcataaaaaaattcaccaaaca	1502–1474	9
Tsol cox1/R1370	acagtagacaccattttaattcaatt	1370–1345	PS
Tsol cox1/R1280	cattttcattatgttatgtcattagggtc	1282–1253	PS
Tsol cox1/R1150	atgacataacataatgaaaatgagca	1150–1125	PS
Tsol cox1/R1050	aaatgtaaacaatactataaacga	1053–1029	PS
Taenia cox R4	attatcatagtaacagaactaaaaaatac	935–907	9
Tsol cox1/830	agcaaacaataacccataa	828–809	PS
Taenia cox R3	gatgaccaaaaaatcaaaacatatgttg	721–694	9
Tsol cox1/R600	aggtaaagtaaccaataacaagatag	600–575	PS
Tsol cox1/R500	atgtacatataaaattaatagaactaaa	505–478	PS
Tsol cox1/R400	taaaggtgggtaaaaagtcc	399–380	PS
Tsol cox1/R280	atccgataatcctcttatca	282–263	PS
Tsol cox1/R190	ttagtaatcaaaaatttataacaatcc	188–162	PS

PS = present study.

mtDNA sequences, *T. solium* can be divided into two genotypes, Asian and African/Latin American.[8] Histological sections were processed by using xylene and ethanol for paraffin removal and were then rehydrated. DNA was extracted with DNeasy tissue kit (Qiagen, Germany). Because of the degradation of DNA, it is difficult to obtain long-fragment DNA from formalin-fixed materials. So we performed polymerase chain reaction (PCR) using primer pairs that can amplify 100 to 200 base pair (bp) fragments. Some of the primers were already reported elsewhere[9] and others were newly designed for the present study (Table 1). PCR products were directly sequenced and the obtained sequences were concatenated and compared with *cox1* sequences available in GenBank database. The following sequences (with GenBank accession numbers) were used for comparison: China 1 (AB066485), China 2 (AB066486), Korea (DQ089663), Thailand (AB066487), Papua (= former Irian Jaya) (AB066488), Bali (AB271234), India (AB066489), Mexico/Peru/Cameroon (AB066490), Ecuador/Bolivia (AB066491), Brazil (AB066492), Tanzania/Mozambique (AB066493). Because no *cox1* sequence of *T. solium* from Nepal, one of the countries where the patient had stayed before (1978–1979, 1984–1986), had been deposited to the database, we collected cysticerci from pigs in three different localities of Nepal (Sunsari, Moranga, and Kathmandu) for *cox1* analysis. One cysticercus was selected from each locality and processed as described in the previous study.[8] As a result, we obtained a partial *cox1* sequence (1570 bp) from the patient (AB494702) and two slightly different sequences of complete *cox1* (1620 bp) from Nepal (Nepal 1: Sunsari, AB491985, Nepal 2: Moranga and Kathmandu, AB491986). The sequence from the patient was identical to one of the two Nepal haplotypes, which was obtained from Sunsari direct. To estimate the genealogical relationship among the haplotypes in the world, we conducted the parsimony network analysis based on the partial *cox1* sequences (1570 bp) with the program TCS version 1.2.[10] As a result, the haplotypes were clearly divided into two geographical groups as previously reported,[8] and the one from the patient was placed into the Asian group (Figure 1). The haplotype from Bali was not included in the haplotype network analysis because only a short sequence (1188 bp) was available in GenBank; however, it was obviously different from all of the others. The result strongly suggests that the patient became infected with *T. solium* not in Indonesia, but in Nepal, an endemic country for cysticercosis.[11] Our result also indicates that he acquired infection before 1986, the last visit to Nepal, and it means that cysticercus had survived in the patient's brain for at least 10 years.

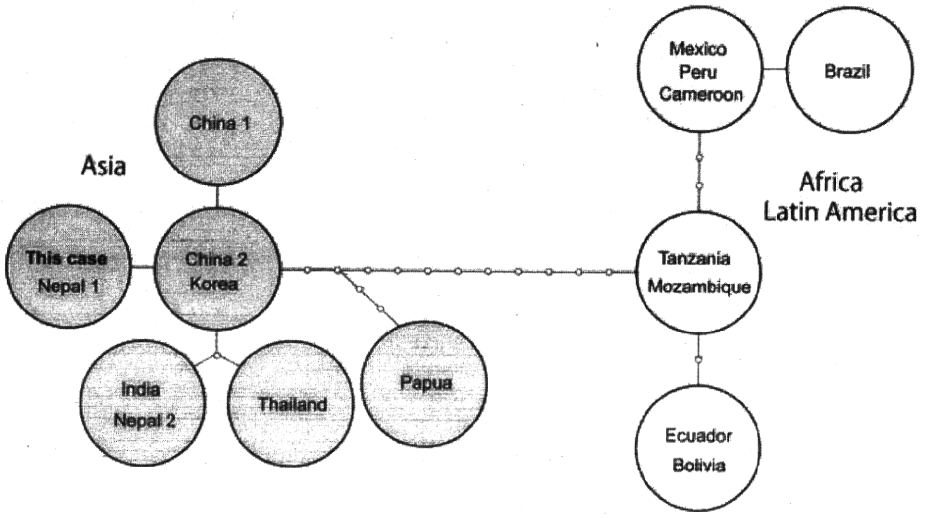

Figure 1 The statistical parsimony networks of *cox1* haplotypes based on 1570 bp sequences. Circles represent haplotypes and each node indicates one mutation step.

Conclusion

As NCC is caused by ingesting the eggs of *T. solium*, even only one teniasis patient can easily disperse this serious disease. Therefore, it is important for disease control and prevention to know where, when, and how the patient acquired NCC, especially in nonendemic countries. As shown in the present study, molecular analysis using *cox1* gene can be a powerful tool for assessing where the patient became infected with *T. solium*, especially in the case of patients who traveled to multiple endemic countries, or who had never visited such contaminated regions. Another mtDNA gene ie, cytochrome *b* has also been demonstrated to serve as a marker for molecular subtyping of *T. solium*.[8] However, we still lack the genetic information from many of the endemic regions. A more globally extensive collection of the specimens, from both domestic pigs and human patient, is needed to make a more detailed genotype map of *T. solium*.

Acknowledgments

This work was supported by the Asia/Africa Science Platform Fund (2006-2011) and International Joint Research Project (17256002, 21256003) from the Japan Society for the Promotion of Science to A. Ito.

Declaration of Interests

The authors state they have no conflicts of interest to declare.

References

1. Schantz PM, Moore AC, Munoz JL, et al. Neurocysticercosis in an Orthodox Jewish community in New York City. N Engl J Med 1992; 327:692–695.
2. Ibrahim N, Azman Ali R, Basri H, et al. Neurocysticercosis in a Malaysian Muslim. Neurol J Southeast Asia 2003; 8:45–48.
3. Schantz PM, Wilkins PP, Tsang VCW. Immigrants, imaging and immunoblots: the emergence of neurocysticercosis as a significant public health problem. In: Scheld WM, Craig WA, Hughes JM, eds. Emerging infections. Vol 2, Washington, DC: ASM Press, 1998: 213–242.
4. Ito A, Nakao M, Wandra T. Human taeniasis and cysticercosis in Asia. Lancet 2003; 362:1918–1920.
5. Yuzawa I, Kawano N, Suzuki S, et al. A case of solitary cerebral cysticercosis. Jpn J Neurosurg 2000; 9:364–369.
6. Ito A, Nakao M, Ito Y, et al. Neurocysticercosis case with a single cyst in the brain showing dramatic drop in specific antibody titers within 1 year after curative surgical resection. Parasitol Int 1999; 48:95–99.
7. Murrell KD. Epidemiology of taeniasis and cysticercosis. In: Murrell KD ed. WHO/FAO/OIE Guidelines for the surveillance, prevention and control of taeniosis/cysticercosis. Paris: OIE, 2005:27–43.
8. Nakao M, Okamoto M, Sako Y, et al. A phylogenetic hypothesis for the distribution of two genotypes of the pig tapeworm *Taenia solium* worldwide. Parasitology 2002; 124:657–662.
9. Yamasaki H, Nakao M, Sako Y, et al. Molecular identification of *Taenia solium* cysticercus genotype in the histopathological specimens. Southeast Asian J Trop Med Public Health 2005; 36(Suppl):S131–S134.
10. Clement M, Posada D, Crandall KA. TCS: a computer program to estimate gene genealogies. Mol Ecol. 2000; 9:1657–1659.
11. Amatya BM, Kimula Y. Cysticercosis in Nepal: a histopathologic study of sixty-two cases. Am J Surg Pathol 1999; 23:1276–1279.

International Journal for Parasitology 40 (2010) 379–385

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences ☆

Minoru Nakao [a,*], Tiaoying Li [a,b], Xiumin Han [a,c], Xiumin Ma [a,d,e], Ning Xiao [a,b], Jiamin Qiu [b], Hu Wang [c], Tetsuya Yanagida [a], Wulamu Mamuti [a,d], Hao Wen [e], Pedro L. Moro [f], Patrick Giraudoux [g], Philip S. Craig [h], Akira Ito [a]

[a] *Department of Parasitology, Asahikawa Medical College, Asahikawa, Hokkaido 078-8510, Japan*
[b] *Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu 610041, China*
[c] *Qinghai Province Institute for Endemic Disease Control, Xining, Qinghai 811602, China*
[d] *Department of Parasitology and Microbiology, Xinjiang Medical University, Urumqi 830054, China*
[e] *Xinjiang Hydatid Clinical Research Institute, The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, China*
[f] *Immunization Safety Office, Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*
[g] *Department of Chrono-environment, UMR UFC/CNRS 6249 aff. INRA, Université de Franche-Comté, Besançon, France*
[h] *Cestode Zoonoses Research Group, Bioscience Research Institute and School of Environment and Life Sciences, University of Salford, Great Manchester M5 4WT, UK*

ARTICLE INFO

Article history:
Received 23 July 2009
Received in revised form 14 September 2009
Accepted 15 September 2009

Keywords:
Echinococcus
Mitochondrial DNA
Genetic diversity
Population genetic structure
China

ABSTRACT

The genetic polymorphisms of *Echinococcus* spp. in the eastern Tibetan Plateau and the Xinjiang Uyghur Autonomous Region were evaluated by DNA sequencing analyses of genes for mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor-1 alpha (*ef1a*). We collected 68 isolates of *Echinococcus granulosus* sensu stricto (s.s.) from Xinjiang and 113 isolates of *E. granulosus* s. s., 49 isolates of *Echinococcus multilocularis* and 34 isolates of *Echinococcus shiquicus* from the Tibetan Plateau. The results of molecular identification by mitochondrial and nuclear markers were identical, suggesting the infrequency of introgressive hybridization. A considerable intraspecific variation was detected in mitochondrial *cox1* sequences. The parsimonious network of *cox1* haplotypes showed star-like features in *E. granulosus* s. s. and *E. multilocularis*, but a divergent feature in *E. shiquicus*. The *cox1* neutrality indexes computed by Tajima's *D* and Fu's Fs tests showed high negative values in *E. granulosus* s. s. and *E. multilocularis*, indicating significant deviations from neutrality. In contrast, the low positive values of both tests were obtained in *E. shiquicus*. These results suggest the following hypotheses: (i) recent founder effects arose in *E. granulosus* and *E. multilocularis* after introducing particular individuals into the endemic areas by anthropogenic movement or natural migration of host mammals, and (ii) the ancestor of *E. shiquicus* was segregated into the Tibetan Plateau by colonising alpine mammals and its mitochondrial locus has evolved without bottleneck effects.

1. Introduction

Metacestodes of the dog tapeworm *Echinococcus granulosus* sensu stricto (s.s.) and the fox tapeworm *Echinococcus multilocularis* are highly pathogenic to humans, and cause cystic and alveolar echinococcoses, respectively. Humans become infected through oral ingestion of eggs derived from faeces of canine definitive hosts. Sheep are a main intermediate host for *E. granulosus* s. s., whereas arvicoline rodents serve as intermediate hosts for *E. multilocularis* (Eckert and Deplazes, 2004). Besides the main two species, *Echinococcus equinus*, *Echinococcus ortleppi*, *Echinococcus canadensis*, *Echinococcus felidis*, *Echinococcus shiquicus*, *Echinococcus vogeli* and *Echinococcus oligarthrus* have been regarded as valid by recent phylogenetic studies (Xiao et al., 2005; Nakao et al., 2007; Hüttner et al., 2008). However, the species status of *E. canadensis* is still debatable (Thompson, 2008).

In China, *E. granulosus* s. s. and *E. multilocularis* are widespread in western, northern and central parts of the country (Wang et al., 2008), and hyperendemic foci exist within pastoral areas of the eastern Tibetan Plateau (Schantz et al., 2003; Li et al., 2008) and the Xinjiang Uyghur Autonomous Region (Wang et al., 2001). Both endemic regions are geographically separated by the Kunlum mountains and the Taklamakan Desert. The alpine steppe of the Tibetan Plateau supports human pastoral activity for the raising of yak and sheep. In Xinjiang, nomads and semi-nomads keep

☆ Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under the Accession Nos. AB491414–AB491471.

* Corresponding author. Tel.: +81 166 68 2422; fax: +81 166 68 2429.
E-mail address: nakao@asahikawa-med.ac.jp (M. Nakao).

0020-7519/$36.00 © 2009 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2009.09.006

livestock on low-altitude grassland. This type of sheep husbandry system, including the use of pastoral dogs, is essential to maintain the synanthropic cycle of *E. granulosus* s. s. In contrast, the rodent fauna of grassland and the migration of foxes are key factors in establishing the endemic foci of *E. multilocularis* (Giraudoux et al., 2006). Human infections with *E. multilocularis* are extremely frequent in the Tibetan communities of Sichuan province (Craig, 2006), and the role of dogs in the communities is important to infections (Wang et al., 2006). Thus, the life-cycle of *E. multilocularis* is altered to be synanthropic in hyperendemic areas.

Species of *Echinococcus* prevailing in China have been clarified by molecular taxonomic studies using mtDNA markers (McManus et al., 1994; Zhang et al., 1998; Yang et al., 2005; Bart et al., 2006; Xiao et al., 2004, 2005, 2006; Ma et al., 2008; Li et al., 2008). The molecular identification of *Echinococcus* isolates from various origins showed the following host–parasite relationships in China: (i) domestic mammals (sheep, cattle, goats, yaks and dogs) for *E. granulosus* s. s., (ii) domestic mammals (camels, cattle and dogs) for *E. canadensis* (G6 genotype), (iii) wildlife (voles, hares, pikas, red foxes and Tibetan foxes) and domestic mammals (dogs) for *E. multilocularis* and (iv) wildlife (pikas and Tibetan foxes) for *E. shiquicus*. Human infections have been confirmed for all species except *E. shiquicus*. Interestingly, *E. canadensis* G6 has been found only in Xinjiang (Zhang et al., 1998) and *E. shiquicus* seems to be restricted in the Tibetan Plateau (Xiao et al., 2005). All of the epidemiological information provides a basis to consider the natural history of *Echinococcus* in China. Previous studies demonstrated that intraspecific mtDNA variations occurred in *E. granulosus* s. s. (Yang et al., 2005; Bart et al., 2006; Ma et al., 2008), *E. multilocularis* (Yang et al., 2005) and *E. shiquicus* (Xiao et al., 2005). However, the genetic populations of *Echinococcus* spp. in China have never been characterised in an evolutionary context.

In this study, the genetic diversities of *E. granulosus* s. s., *E. multilocularis* and *E. shiquicus* were explored by using mtDNA and nDNA markers. We sampled the isolates of *E. granulosus* s. s. from Xinjiang and the isolates of all three species from the eastern Tibetan Plateau. The main purpose of this study was to evaluate the population genetic structures of the three species. The resultant data and epidemiological information enabled us to suggest evolutionary hypotheses on how the parasites have spread in China.

2. Materials and methods

2.1. Isolates collected

An *Echinococcus* isolate was defined as a unilocular cyst or a separated alveolar cyst from an intermediate host. During the period from 2002 to 2007, the larval isolates of *Echinococcus* spp. from various hosts were collected in the eastern Tibetan Plateau (Qinghai and Sichuan provinces) and the Xinjiang Uyghur Autonomous Region. Table 1 summarises the number and origin of isolates examined in the two localities. For *E. granulosus* s. s., 113 isolates in the highlands and 68 isolates in the lowlands were treated as two separate populations. On the other hand, 49 isolates of *E. multilocularis* and 34 isolates of *E. shiquicus* were obtained only from the highlands. One isolate in the lowlands was identified as *E. canadensis* G6 genotype. In addition, 57 isolates of *E. granulosus* s. s. in Peru (Moro et al., 2009) served as a foreign control for population genetic analyses. The species identification of those isolates was validated by DNA sequencing described below.

2.2. PCR amplification and sequencing

The genomic DNA of each isolate was prepared from ethanol-preserved larval cysts by using a DNeasy blood and tissue kit (Qiagen), and used as a template for PCR. Partial fragments of a mitochondrial gene for cytochrome *c* oxidase subunit 1 (*cox1*) and a nuclear gene for elongation factor-1 alpha (*ef1a*) were amplified by PCR using specific primers reported previously (Nakao et al., 2000; Moro et al., 2009). The PCR mixture was prepared in a 25 μl final volume containing 1 μl template DNA, 200 μM of each dNTP, 0.2 μM of each primer, 0.5 U of Ex-*Taq* polymerase (Takara) and the manufacturer-supplied reaction buffer. Thermal reactions were performed for 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. Amplified DNA fragments were purified with QIAquick spin columns (Qiagen), and sequenced directly with a BigDye terminator cycle sequencing kit (Applied Biosystems). The resultant sequence ladders were read by an ABI PRISM 377 genetic analyzer (Applied Biosystems).

2.3. Data analysis

In each species of *Echinococcus*, multiple alignments in NEXUS format were made manually by editing the plain text files of nucleotide sequences. Amino acid sequences were inferred from the nucleotide sequences by echinoderm mitochondrial genetic code (Nakao et al., 2000; Telford et al., 2000) or standard genetic code. Percentage divergence values of nucleotide sequences were determined using the MEGA4 package (Tamura et al., 2007) using Kimura's two parameter model (Kimura, 1980) with a γ-shaped parameter ($\alpha = 0.5$). The identification of haplotypes and the drawing of their networks were executed by TCS 1.2 software (Clement et al., 2000) using statistical parsimony (Templeton et al., 1992). The network estimation was run at a 95% connection limit.

Population diversity indexes (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated using DnaSP 4.5

Table 1
Number of *Echinococcus* isolates used for this study.

Species and localities	Origins of larval isolates						
	Human	Sheep	Yak	Rodent[a]	Hare[b]	Pika[c]	Total
Echinococcus granulosus							
Qinghai & Sichuan	37	57	19	0	0	0	113
Xinjiang	54	14	0	0	0	0	68
Total	91	71	19	0	0	0	181
Echinococcus multilocularis							
Qinghai & Sichuan	20	0	0	26	1	2	49
Echinococcus shiquicus							
Qinghai & Sichuan	0	0	0	0	0	34	34
Echinococcus canadensis (G6)							
Xinjiang	1	0	0	0	0	0	1

[a] *Microtus fuscus, Microtus limnophilus* and *Cricetulus kamensis.*
[b] *Lepus oiostolus.*
[c] *Ochotona curzoniae.*

software (Rozas et al., 2003). The population genetics package Arlequin 3.1 (Excoffier et al., 2005) was employed to calculate the neutrality indexes of Tajima's *D* (Tajima, 1989) and Fu's Fs (Fu, 1997). The degree of gene flow between two populations was estimated using a pairwise fixation index (Fst) as determined by the Arlequin package. The three geographic populations of *E. granulosus* s. s. from Xinjiang, the eastern Tibetan Plateau and Peru (out of China) were used to compute the Fst values.

3. Results

3.1. Variations in nucleotide sequences

In our targeted regions of *Echinococcus* DNA, deletion or insertion mutations were not observed, even in different species. The total numbers of nucleotides examined were therefore stable in mitochondrial *cox1* (789 sites) and nuclear *ef1a* (656 sites). The *cox1* sequences could be amplified in all of the isolates of *E. granulosus* s. s. ($n = 181$), *E. multilocularis* ($n = 49$), *E. shiquicus* ($n = 34$) and *E. canadensis* G6 ($n = 1$). However, the PCR positive rate of *ef1a* was relatively lower than that of *cox1*, probably due to the low copy number of the nuclear gene. Each *Echinococcus* sp. retained the species-specific nucleotide sequences of *cox1* and *ef1a* (Table 2).

Considerable intraspecific variations were detected only in the mtDNA sequences of *cox1* (Table 1), indicating its primary use for population genetic analyses. Synonymous substitutions exceeded non-synonymous substitutions in the *cox1* sequences of *E. granulosus* s. s. and *E. shiquicus*. Out of all of the point mutations, 24 sites (49.0%) of *E. granulosus* s. s., one site (25.0%) of *E. multilocularis* and 11 sites (64.7%) of *E. shiquicus* were parsimony informative. Relatively to *E. shiquicus*, singleton substitutions were abundant in *E. granulosus* s. s. and *E. multilocularis*. The pairwise divergence of the *cox1* sequences was computed among individual isolates at an intraspecific level. The maximum values of the divergence were 0.9% in *E. granulosus* s. s., 0.3% in *E. multilocularis* and 1.5% in *E. shiquicus*. The autochthonous species *E. shiquicus* appeared to have the most variable mtDNA.

3.2. Haplotype networks

In *E. granulosus* s. s., 43 mtDNA haplotypes were found in 181 isolates from Xinjiang and the eastern Tibetan Plateau (Qinghai and Sichuan). To discern a genealogical relationship among the haplotypes, we constructed a statistical parsimony network. As shown in Fig. 1, each of the two regions possessed geographically specific haplotypes. The network, however, showed a star-like expansion, and one common haplotype (G01) occupied the centre of the network. The numbers of mutational steps between the common haplotype and the others ranged from one to five, and the frequency of the common haplotype was 53.6% in the population. A similar star-like network was observed in the Peruvian population of *E. granulosus* s. s. (Fig. 2A). Five mtDNA haplotypes were detected in the 57 Peruvian isolates (Moro et al., 2009), but the majority of the isolates (93.0%) belonged to the haplotype G01, which was the most common in the Chinese populations. A single-nucleotide variation was identified between members of the five haplotypes, three of which were geographically specific to Peru.

We detected five mtDNA haplotypes in 49 isolates of *E. multilocularis* from the eastern Tibetan Plateau. These were illustrated as a star-like network with one major haplotype (M01), which comprised 89.8% of the isolates examined (Fig. 2B). All variations found in the five haplotypes were single-nucleotide polymorphisms. As opposed to the convergent networks of *E. granulosus* s. s. and *E. multilocularis*, a divergent network was found in *E. shiquicus* (Fig. 2C). Although 10 mtDNA haplotypes were detected in 34 isolates of *E. shiquicus*, major haplotypes were absent in the population. The maximum number of mutational steps was 13 in the network of *E. shiquicus*.

3.3. Diversity and neutrality indexes

Diversity indexes for *Echinococcus* populations in each locality were calculated using the data set of *cox1* (Table 3). In the Chinese populations of *Echinococcus* spp., both values of haplotype and nucleotide diversities were the highest in *E. shiquicus* but the lowest in *E. multilocularis*. In the case of *E. granulosus* s. s., the Peruvian population showed the lowest values compared with the Chinese populations. The high levels of haplotype diversity were kept in the Chinese populations of *E. granulosus* s. s., but their nucleotide diversity was relatively low because of the richness of single-nucleotide substitutions.

Neutrality indexes calculated by Tajima's *D* and Fu's Fs tests are also shown in Table 3. The highly negative values were recorded in the Chinese populations of *E. granulosus* s. s. and *E. multilocularis*, indicating a significant deviation from neutrality. The Peruvian population of *E. granulosus* s. s. also showed significant negative values. By contrast, relatively low positive values were obtained in the population of *E. shiquicus*, which kept highly polymorphic mtDNA.

3.4. Fixation index for the populations of E. granulosus s. s

Using the data set of mtDNA, the values of the pairwise fixation index (Fst) were computed to estimate the degree of gene flow among three geographic populations of *E. granulosus* s. s in China and Peru (Table 4). Since one common haplotype existed predominantly in the three localities, the Fst values between the populations were very small, ranging from 0.036 to 0.009. These low values implied that the populations were not genetically differentiated from one another.

4. Discussion

Echinococcoses caused by *E. granulosus* s. s. and *E. multilocularis* lead to considerable social and economic losses in the endemic communities of the eastern Tibetan Plateau (Budke et al., 2005). Furthermore, *E. shiquicus* has been recently discovered in the plateau (Xiao et al., 2005). Information on the population genetic structures of these sympatric species is necessary to better understand the process of intra- and interspecific gene flows, and may provide a foundation for future epidemiological studies on the transmission dynamics of the parasites. In the present study, mtDNA revealed the basic structures of *Echinococcus* populations in the eastern Tibetan Plateau.

Table 2
Number of nucleotide substitutions in mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor-1 alpha (*ef1a*) genes amplified from *Echinococcus* spp. in China.

Species	*cox1* (789)			*ef1a* (656)		
	n	*S*	NS	*n*	*S*	NS
Echinococcus granulosus	181	30	19	122	0	0
Echinococcus multilocularis	49	1	3	38	0	0
Echinococcus shiquicus	34	16	1	30	3	0

Number of the total nucleotide sites examined is shown in parentheses. Abbreviations are number of isolates examined (*n*), synonymous substitutions (*S*) and non-synonymous substitutions (NS).

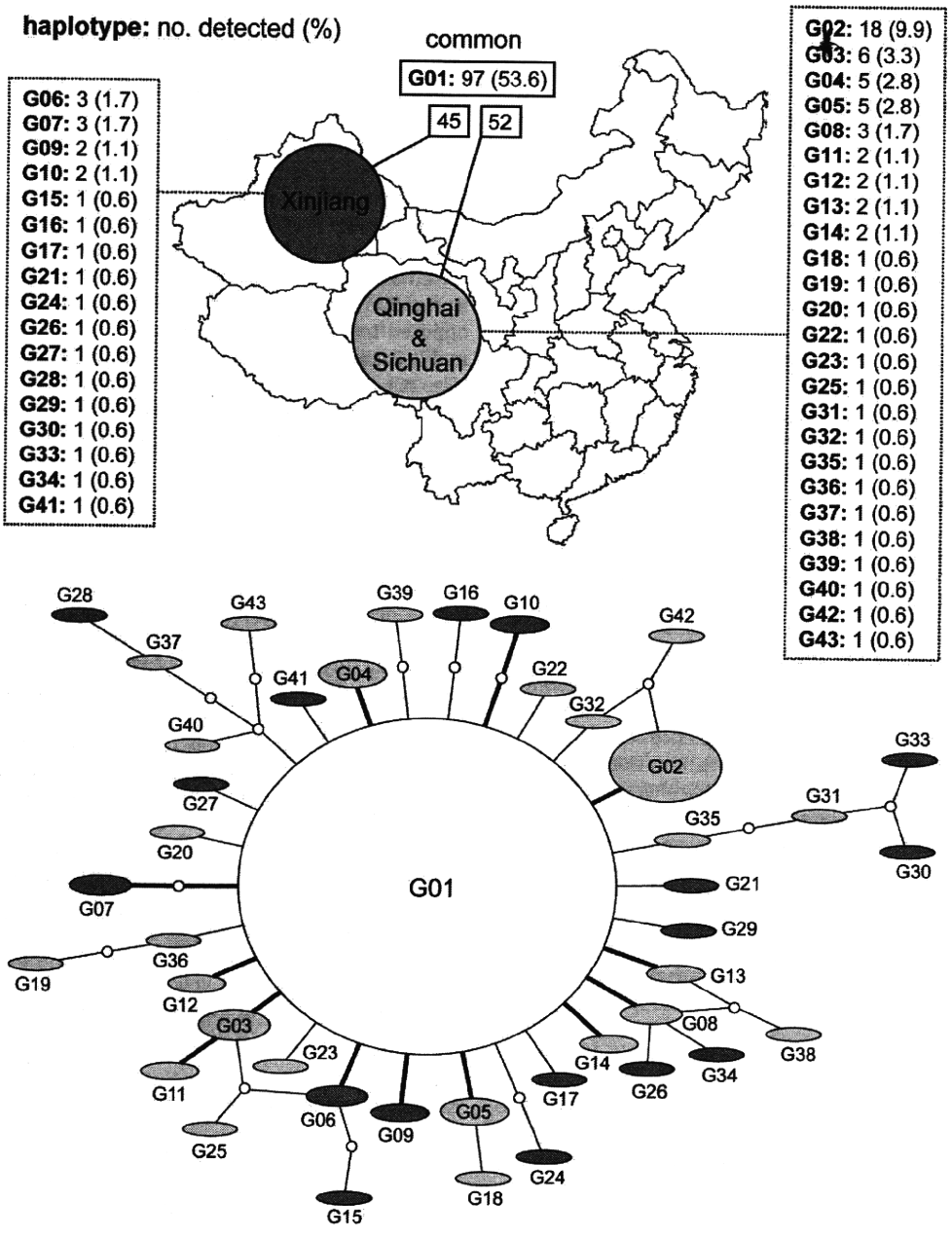

Fig. 1. Frequencies of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) haplotypes in Chinese *Echinococcus granulosus* and their network based on statistical parsimony. In the network, the size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. The haplotypes whose frequencies are more than 1% are connected with bold lines. Dark ovals represent the haplotypes found in Xinjiang, whereas the localities of grey ovals are Qinghai and Sichuan. A white oval shows the common haplotype.

We chose the protein-coding gene *cox1* as a marker for *Echinococcus* mtDNA. In other organisms, the mitochondrial control region has been generally used to infer genealogical relationships. However, the corresponding mtDNA regions of *Echinococcus* spp. contain highly repetitive sequences, which are unsuitable for phylogenetic studies (Nakao et al., 2007). The *cox1* gene of *Echinococcus* spp. has already been shown to be a promising candidate for the classification of intra- and interspecific variants even in the short sequence (366 nucleotide sites) (Bowles et al., 1992). In this study, we determined the relatively long sequence of *cox1* (789 nucleotide sites), and detected a sufficient number of haplotypes to analyse the population genetic structures of each species. The haplotypes are only loosely correlated with the intraspecific genotypes of *E. granulosus s. s.* (G1, G2 and G3) and *E. multilocularis* (M1 and M2) defined by Bowles et al. (1992). The negative selection of the *cox1* for the purging of deleterious mutations has been demonstrated by the codon-based *Z*-test (Tamura et al., 2007) using the corresponding sequences of *E. granulosus s. s.*, *E. multilocularis*, *E. equinus*, *E. ortleppi*, *E. canadensis*, *E. felidis*, *E. vogeli* and *E. oligarthrus* (M. Nakao, unpublished data). The fragments of the nuclear gene *ef1a* were also sequenced in this study, but their species-specific sequences were not polymorphic at an intraspecific level. The results of species identification by the mitochondrial and nuclear markers were identical, suggesting the infrequency of introgressive hybridization among the sympatric species.

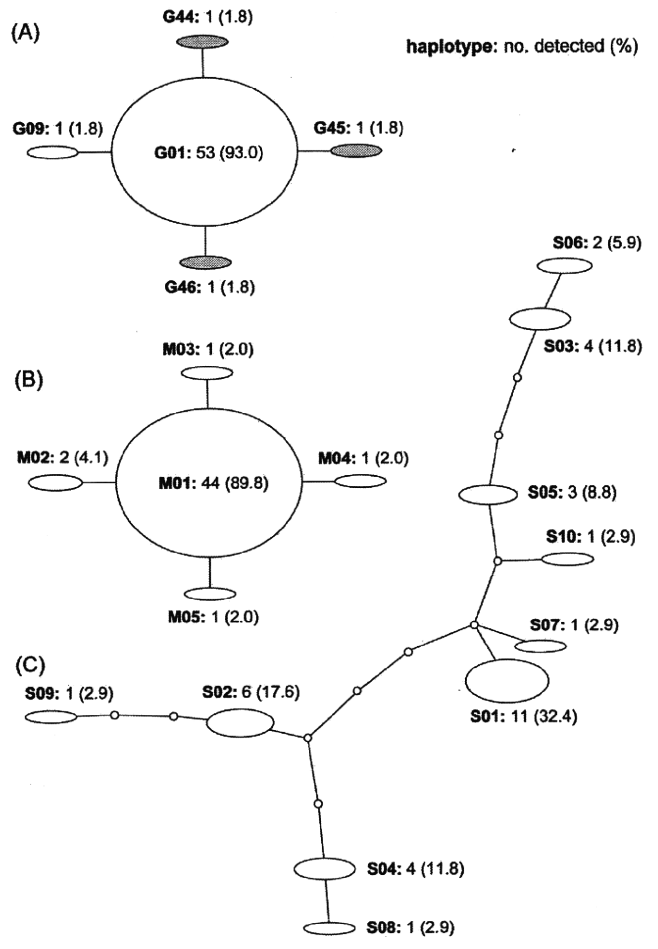

Fig. 2. The statistical parsimony networks of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) haplotypes in *Echinococcus* spp. The size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. (A) The Peruvian population of *Echinococcous granulosus* sensu stricto. Grey ovals represent the haplotypes specific to Peru. (B) The Tibetan population of *Echinococcus multilocularis*. (C) The Tibetan population of *Echinococcus shiquicus*.

The *cox1* haplotypes of *E. granulosus* s. s. found in this study did not show an apparent phylogeographic structuring in China. The parsimony network analysis revealed that the haplotypes exhibit a star-like expansion from a main founder haplotype, suggesting that the populations of eastern Tibet and Xinjiang are not fully differentiated from each other. It is noteworthy that the same founder was predominant in the Peruvian population. It seems unlikely that mutations of the founder haplotype are advantageous because the amino acid sequence deduced from the founder is the same as those from other minor haplotypes. The common genetic structure between geographically unrelated populations enables us to speculate that one particular lineage of *E. granulosus* s. s. is widespread globally. The genetic non-differentiation between the local populations is also demonstrated by extreme low values of the fixation index Fst. Furthermore, the significant negative values of the neutrality indexes Tajima's *D* and Fu's Fs suggest that bottleneck events might occur in the recent past. It is most likely that demographic expansions of the parasite occurred after introducing particular individuals into the endemic areas by anthropogenic movements of host mammals (sheep and dogs).

It is assumed that *E. granulosus* s. s. was introduced into South America from Europe through livestock importation after the colonial period. The higher values of haplotype and nucleotide diversities in the Chinese populations of *E. granulosus* s. s. suggest that China historically preceded Peru in the time of initial founder introduction. One could speculate that bottleneck events might also occur in the ancestral population of *E. granulosus* s. s. during the colonisation of domestic sheep as an intermediate host. Archaeological and genetic evidence suggest that sheep were domesticated in the Ancient Near East (Pedrosa et al., 2005), but the genealogical survey of Chinese domestic sheep showed the possibility that additional domestication events occurred independently in other regions (Chen et al., 2006). The lack of archaeoparasitological data does not permit us to infer how and when the parasite invaded China. However, the Ancient Near East is one possible candidate for the cradle of *E. granulosus* s. s. The population genetic structures of *E. granulosus* s. s. should be compared in various endemic areas to clarify its ancestral origin and the process of its worldwide dispersal.

Our previous study has already indicated the rarity of mtDNA polymorphism in the Tibetan population of *E. multilocularis* (Xiao et al., 2005). The present study furthermore revealed that a particular *cox1* sequence was positioned as a basal haplotype, suggesting that a founder effect arose in the population. Our recent phylogeographic study on *E. multilocularis* showed that the worldwide iso-

Table 4
Pairwise fixation index (Fst values) between *Echinococcus granulosus* sub-populations calculated from the nucleotide data set of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene.

	1	2
1. Qinghai & Sichuan		
2. Xinjiang	0.031[a]	
3. Out of China (Peru)	0.036[a]	0.009

[a] Significant *P* values ($P < 0.01$).

Table 3
Diversity and neutrality indexes for *Echinococcus* populations calculated from the nucleotide data set of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene.

Species and localities	Diversity				Neutrality	
	n	Hn	Hd ± S.D.	π ± S.D.	*D*	Fs
Echinococcus granulosus						
Qinghai & Sichuan	113	26	0.760 ± 0.038	0.0017 ± 0.0002	−2.323[a]	−26.023[a]
Xinjiang	68	18	0.562 ± 0.073	0.0015 ± 0.0003	−2.456[a]	−15.762[a]
Total (China)	181	43	0.702 ± 0.038	0.0017 ± 0.0002	−2.536[a]	−61.569[a]
Out of China (Peru)	57	5	0.137 ± 0.062	0.0002 ± 0.0001	−1.849[a]	−5.889[a]
Echinococcus multilocularis						
Qinghai & Sichuan	49	5	0.195 ± 0.075	0.0003 ± 0.0001	−1.765[a]	−4.788[a]
Echinococcus shiquicus						
Qinghai & Sichuan	34	10	0.847 ± 0.040	0.0055 ± 0.0004	0.164	0.258

Abbreviations are number of isolates examined (*n*), number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π), Tajima's *D* (*D*) and Fu's Fs (Fs).
[a] Significant *P* values ($P < 0.01$).

lates were classified into European, Asian and North American clades except the Inner Mongolia isolates from the corsac fox *Vulpes corsac* (Nakao et al., 2009). The geographic clustering indicates a possibility that genetic changes occurred in *E. multilocularis* after the fragmentation of the population during the Pleistocene ice ages. The red fox *Vulpes vulpes*, which has a flexible ability to adapt to various environments, extended its distributional range in the Holarctic region, and might play an essential role in introducing *E. multilocularis* into new areas. It seems likely that an epidemic of the parasite in the eastern Tibetan Plateau was initiated by natural migration of red foxes in the recent past.

The Tibetan indigenous species *E. shiquicus* showed a quite different pattern of population genetic structure when compared with *E. granulosus* s. s. and *E. multilocularis*. Statistical neutrality tests and haplotype network analyses suggest a possibility that the mitochondrial locus of *E. shiquicus* has evolved without bottleneck effects. Our previous report clarified that *E. shiquicus* utilises the Tibetan fox *Vulpes ferrilata* as a definitive host and the plateau pika *Ochotona curzoniae* as an intermediate host (Xiao et al., 2005). Both the autochthonous mammals are adapted to the high altitude steppe but do not survive in lowlands. We can therefore consider that *E. shiquicus* has been segregated in the plateau since the parasite's ancestor colonised the alpine mammals. The lasting geographic segregation seems to be a cause for extraordinary richness of polymorphism in Tibetan *E. shiquicus*.

Diploid organisms having a mixed sexual and asexual reproduction system show different patterns from theoretical population genetic models (Prugnolle et al., 2005). The adult tapeworms of *Echinococcus* are hermaphroditic, and self-fertilisation mainly occurs in the small intestine of canine definitive hosts (Haag et al., 1999). The larvae furthermore proliferate asexually in the viscera of intermediate hosts, and the clonal offspring develop into adults in a definitive host. The biphasic reproduction of *Echinococcus* spp. may strongly affect their population genetic structures and promote a very low genetic variability of nuclear loci. In this study we used a haploid maternally inherited mtDNA marker to examine the population genetic structure of *Echinococcus* because of the lack of appropriate nuclear markers. A panel of single-locus nuclear markers is required for further population genetic studies to elucidate the evolutionary backgrounds of *Echinococcus* worldwide.

Acknowledgments

This study was supported by the US National Institutes of Health Funds (the NIH/NSF Ecology and Infectious Diseases Program, # TWO 1565, Principal Investigator: P.S. Craig), the Japan Society for the Promotion of Science (JSPS) (17256002, 21256003) and JSPS-Asia/Africa Science Platform Fund (2006–2011) to A. Ito. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Fogarty International Center or the National Institutes of Health. The authors thank many colleagues and field workers who collected the isolates of *E. granulosus* s. s., *E. multilocularis* and *E. shiquicus* in China.

References

Bart, J.M., Abdukader, M., Zhang, Y.L., Lin, R.Y., Wang, Y.H., Nakao, M., Ito, A., Craig, P.S., Piarroux, R., Vuitton, D.A., Wen, H., 2006. Genotyping of human cystic echinococcosis in Xinjiang, PR China. Parasitology 133, 571–579.

Bowles, J., Blair, D., McManus, D.P., 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol. Biochem. Parasitol. 54, 165–173.

Budke, C.M., Jiamin, Q., Qian, W., Torgerson, P.R., 2005. Economic effects of echinococcosis in a disease-endemic region of the Tibetan Plateau. Am. J. Trop. Med. Hyg. 73, 2–10.

Chen, S.Y., Duan, Z.Y., Sha, T., Xiangyu, J., Wu, S.F., Zhang, Y.P., 2006. Origin, genetic diversity, and population structure of Chinese domestic sheep. Gene 376, 216–223.

Clement, M., Posada, D., Crandall, K., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1660.

Craig, P.S., 2006. Epidemiology of human alveolar echinococcosis in China. Parasitol. Int. 55, S221–S225.

Eckert, J., Deplazes, P., 2004. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin. Microbiol. Rev. 17, 107–135.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. Online 1, 47–50.

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915–925.

Giraudoux, P., Pleydell, D., Raoul, F., Quéré, J.P., Wang, Q., Yang, Y., Vuitton, D.A., Qiu, J., Yang, W., Craig, P.S., 2006. Transmission ecology of *Echinococcus multilocularis*: what are the ranges of parasite stability among various host communities in China? Parasitol. Int. 55, S237–S246.

Haag, K.L., Araújo, A.M., Gottstein, B., Siles-Lucas, M., Thompson, R.C., Zaha, A., 1999. Breeding systems in *Echinococcus granulosus* (Cestoda; Taeniidae): selfing or outcrossing? Parasitology 118, 63–71.

Hüttner, M., Nakao, M., Wassermann, T., Siefert, L., Boomker, J.D., Dinkel, A., Sako, Y., Mackenstedt, U., Romig, T., Ito, A., 2008. Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. Int. J. Parasitol. 38, 861–868.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Li, T., Ito, A., Nakaya, K., Qiu, J., Nakao, M., Zhen, R., Xiao, N., Chen, X., Giraudoux, P., Craig, P.S., 2008. Species identification of human echinococcosis using histopathology and genotyping in northwestern China. Trans. R. Soc. Trop. Med. Hyg. 102, 585–590.

Ma, S.M., Maillard, S., Zhao, H.L., Huang, X., Wang, H., Geng, P.L., Bart, J.M., Piarroux, R., 2008. Assessment of *Echinococcus granulosus* polymorphism in Qinghai province, People's Republic of China. Parasitol. Res. 102, 1201–1206.

McManus, D.P., Ding, Z., Bowles, J., 1994. A molecular genetic survey indicates the presence of a single, homogeneous strain of *Echinococcus granulosus* in northwestern China. Acta Trop. 56, 7–14.

Moro, P., Nakao, M., Ito, A., Schantz, P.M., Cavero, C., Cabrera, L., 2009. Molecular identification of *Echinococcus* isolates from Peru. Parasitol. Int. 58, 184–186.

Nakao, M., McManus, D.P., Schantz, P.M., Craig, P.S., Ito, A., 2007. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitology 134, 713–722.

Nakao, M., Sako, Y., Yokoyama, N., Fukunaga, M., Ito, A., 2000. Mitochondrial genetic code in cestodes. Mol. Biochem. Parasitol. 111, 415–424.

Nakao, M., Xiao, N., Okamoto, M., Yanagida, T., Sako, Y., Ito, A., 2009. Geographic pattern of genetic variation in the fox tapeworm *Echinococcus multilocularis*. Parasitol. Int. 58, 384–389.

Pedrosa, S., Uzun, M., Arranz, J.J., Gutiérrez-Gil, B., San Primitivo, F., Bayón, Y., 2005. Evidence of three maternal lineages in Near Eastern sheep supporting multiple domestication events. Proc. Biol. Sci. 272, 2211–2217.

Prugnolle, F., Liu, H., Meeûs, T., Balloux, F., 2005. Population genetics of complex life-cycle parasites: an illustration with trematodes. Int. J. Parasitol. 35, 255–263.

Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.

Schantz, P.M., Wang, H., Qiu, J., Liu, F.J., Saito, E., Emshoff, A., Ito, A., Roberts, J.M., 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.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.. Mol. Biol. Evol. 24, 1596–1599.

Telford, M.J., Herniou, E.A., Russell, R.B., Littlewood, D.T., 2000. Changes in mitochondrial genetic codes as phylogenetic characters: two examples from the flatworms. Proc. Natl. Acad. Sci. USA 97, 11359–11364.

Templeton, A.R., Crandall, K.A., Sing, C.F., 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132, 619–633.

Thompson, R.C.A., 2008. The taxonomy, phylogeny and transmission of *Echinococcus*. Exp. Parasitol. 119, 439–446.

Wang, Q., Qiu, J., Yang, W., Schantz, P.M., Raoul, F., Craig, P.S., Giraudoux, P., Vuitton, D.A., 2006. Socioeconomic and behavior risk factors of human alveolar echinococcosis in Tibetan communities in Sichuan, People's Republic of China. Am. J. Trop. Med. Hyg. 74, 856–862.

Wang, Y.H., Rogan, M.T., Vuitton, D.A., Wen, H., Bartholomot, B., Macpherson, C.N., Zou, P.F., Ding, Z.X., Zhou, H.X., Zhang, X.F., Luo, J., Xiong, H.B., Fu, Y., McVie, A., Giraudoux, P., Yang, W.G., Craig, P.S., 2001. Cystic echinococcosis in semi-nomadic pastoral communities in north-west China. Trans. R. Soc. Trop. Med. Hyg. 95, 153–158.

Wang, Z., Wang, X., Liu, X., 2008. Echinococcosis in China, a review of the epidemiology of *Echinococcus* spp.. Ecohealth 5, 115–126.

Yang, Y.R., Rosenzvit, M.C., Zhang, L.H., Zhang, J.Z., McManus, D.P., 2005. Molecular study of *Echinococcus* in west-central China. Parasitology 131, 547–555.

Xiao, N., Li, T.Y., Qiu, J.M., Nakao, M., Chen, X.W., Nakaya, K., Yamasaki, H., Schantz, P.M., Craig, P.S., Ito, A., 2004. The Tibetan hare *Lepus oiostolus*: a novel intermediate host for *Echinococcus multilocularis*. Parasitol. Res. 92, 352–353.

Xiao, N., Nakao, M., Qiu, J., Budke, C.M., Giraudoux, P., Craig, P.S., Ito, A., 2006. Dual infection of animal hosts with different *Echinococcus* species in the eastern Qinghai–Tibet plateau region of China. Am. J. Trop. Med. Hyg. 75, 292–294.

Xiao, N., Qiu, J., Nakao, M., Li, T., Yang, W., Chen, X., Schantz, P.M., Craig, P.S., Ito, A., 2005. *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika in China. Int. J. Parasitol. 35, 693–701.

Zhang, L.H., Chai, J.J., Jiao, W., Osman, Y., McManus, D.P., 1998. Mitochondrial genomic markers confirm the presence of the camel strain (G6 genotype) of *Echinococcus granulosus* in north-western China. Parasitology 116, 29–33.

Parasitology International 59 (2010) 70–74

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Evidence of hybridization between *Taenia saginata* and *Taenia asiatica*

Munehiro Okamoto [a,*], Minoru Nakao [b], David Blair [c], Malinee T. Anantaphruti [d], Jitra Waikagul [d], Akira Ito [b]

[a] Department of Parasitology, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Japan
[b] Department of Parasitology, Asahikawa Medical College, Japan
[c] School of Marine and Tropical Biology, James Cook University, Townsville, Queensland, Australia
[d] Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand

ARTICLE INFO

Article history:
Received 22 June 2009
Received in revised form 17 October 2009
Accepted 19 October 2009
Available online 27 October 2009

Keywords:
Taenia asiatica
Taenia saginata
Outcrossing
Hybridization

ABSTRACT

There has long been a debate as to the specific status of the cestode *Taenia asiatica*, with some people regarding it as a distinct species and some preferring to recognize it as a strain of *Taenia saginata*. The balance of current opinion seems to be that *T. asiatica* is a distinct species. In this study we performed an allelic analysis to explore the possibility of gene exchange between these closely related taxa. In total, 38 taeniid tapeworms were collected from humans living in many localities including Kanchanaburi Province, Thailand where the two species are sympatric. A mitochondrial DNA (mtDNA)-based multiplex PCR tentatively identified those parasites as *T. asiatica* ($n=20$) and *T. saginata* ($n=18$). Phylogenetic analyses of a mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene and two nuclear loci, for elongation factor-1 alpha (*ef1*) and ezrin-radixin-moesin (ERM)-like protein (*elp*), assigned all except two individual parasites to the species indicated by multiplex PCR. The two exceptional individuals, from Kanchanaburi Province, showed a discrepancy between the mtDNA and nuclear DNA phylogenies. In spite of their possession of sequences typical of the *T. saginata cox1* gene, both were homozygous at the *elp* locus for one of the alleles found in *T. asiatica*. At the *ef1* locus, one individual was homozygous for the allele found at high frequency in *T. asiatica* while the other was homozygous for the major allele in *T. saginata*. These findings are evidence of occasional hybridization between the two species, although the possibility of retention of ancestral polymorphism cannot be excluded.

1. Introduction

The family Taeniidae consists of only two genera, *Taenia* and *Echinococcus*. These distinctive tapeworms mature in carnivorous mammals worldwide and several species occur in humans, sometimes causing severe disease. There have been many studies on the morphology and genetics of members of these genera, yet there is still debate about the number of species in each genus and the best means of distinguishing them. In *Echinococcus*, molecular studies based largely on mitochondrial but partially on nuclear genes support the recognition of several distinct species [1–6]. Similar studies on members of *Taenia* [7] have also used mitochondrial sequences to aid recognition of species. Under the biological species concept, distinct species are not expected to exchange genes, or to do so very rarely [8]. Evidence for hybridization is often found by study of nuclear loci and comparison of these with mitochondrial data. Introgression (the infiltration of genes from the gene pool of one species into that of another) can be inferred from the finding of mitochondrial sequences typical of one species in an organism with the nuclear alleles of another. Alternatively, hybridization can be demonstrated by the presence of nuclear alleles in a single individual that are typical of more than one species. Evidence of gene exchange between species of *Echinococcus* has been noted [9,10], thus potentially rekindling the debate about species boundaries in this genus. No such study has explored the question of gene exchange between species in the genus *Taenia*. In this paper, we provide the first evidence that this can occur.

Three human *Taenia* species are found in the Asia-Pacific region: *Taenia solium* (pork tapeworm), *Taenia saginata* (beef tapeworm) and *Taenia asiatica* [11]. The larval stages of these *Taenia* species have been identified as *Cysticercus cellulosae*, *Cysticercus bovis* and *Cysticercus viscerotropica*, respectively [12]. Humans may harbor adult worms after consuming raw or under-cooked pork, beef or viscera of swine, respectively, contaminated with metacestodes of these species. It is important to discriminate among these three taxa, since ingestion of *T. solium* eggs by humans may result in neurocysticercosis, a serious public health problem in many areas worldwide [11,13]. *T. saginata* and *T. asiatica* are morphologically very similar, as are their mitochondrial DNA sequences [14]. On the other hand, the two taxa clearly differ in biological features including host specificity and organotropism [12]. Mitochondrial DNA (mtDNA) sequence data are

* Corresponding author. Department of Parasitology, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, 4-101 Koyama-cho Minami, Tottori-shi, Tottori 680-8553, Japan. Tel./fax: +81 857 31 5639.
E-mail address: mokamoto@muses.tottori-u.ac.jp (M. Okamoto).

1383-5769/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2009.10.007

frequently used to distinguish between these species, but nuclear gene markers have not been used previously. In this study, genotyping at two nuclear loci was carried out to explore the possibility of gene exchange between *T. saginata* and *T. asiatica* in a locality where they are sympatric.

2. Materials and methods

2.1. Parasite samples

Adult tapeworms, which were morphologically similar to *T. saginata*, were collected from humans. In the end, 38 samples from 11 nations (Brazil, Ecuador, Ethiopia, Japan, South Korea, Philippines, China, Taiwan, Cambodia, Thailand and Indonesia) were used in this study. Of these, 15 samples were collected from small villages in Kanchanaburi Province, Thailand. These areas are unique, since the three species of human *Taenia* were identified as sympatrically endemic during surveys in 2002–2005 [16].

2.2. DNA preparation

Genomic DNA was individually extracted from mature or immature proglottids using a QIAamp DNA Mini Kit or a DNeasy tissue kit (QIAGEN, Germany) in accordance with the manufacturer's instructions, and then used as a template for polymerase chain reaction (PCR).

2.3. Multiplex PCR for Taenia species identification

Multiplex PCR based on the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene is an easy method for identification of human taeniid cestodes [16–18]. Samples were first screened by this method for the tentative identification of species. According to the results, the code "Tasi" (*T. asiatica*) or "Tsag" (*T. saginata*) was added to the sample ID. It is important to note that this code refers to the identification from the mitochondrial genome.

2.4. DNA sequencing and data analysis

Multiplex PCR yields products of taxon-specific lengths that can be visualized in a gel. For finer genetic discrimination, the complete sequence of the *cox1* gene was obtained for each individual. Partial sequences of two nuclear genes, elongation factor-1-alpha (*ef1*) and ezrin/radixin/moesin-like protein (*elp*), were also obtained. Primer pairs listed in Table 1 were used for the PCR amplification and sequencing of those genes.

PCR was carried out in 15 μl reaction mixtures containing 1 μl template, 200 μM of each dNTP, 0.2 μM of each primer, 0.3 U of *Ex Taq* polymerase (TaKaRa, Japan) and the manufacturer-supplied reaction buffer. Thermal cycling was performed for 35 cycles of denaturation (94°C for 30 s), annealing (66°C: *cox1*, 60°C: *ef1*, 65°C: *elp*, for 30 s), and extension (72°C for 80–90 s). The PCR products were purified using MinElute PCR Purification Kits (QIAGEN). Direct sequencing was performed with a Dye Terminator Cycle Sequencing Kit and an ABI PRISM 3100 Generic Analyzer (Applied Biosystems, USA). At least two independent PCR products were used for sequencing. Samples that could not be directly sequenced were subjected to cloning using a TOPO TA Cloning Kit (invitrogen, USA), and more than ten clones were sequenced per sample.

DNA sequences were aligned using the CLUSTAL W computer program [19]. Phylogenetic trees were constructed by the neighbor-joining (NJ) method [20] using the MEGA4.0 computer program [21]. Evolutionary distances were computed using the Maximum Composite Likelihood Method [22]. Each of the phylogenetic trees was evaluated using a bootstrap test based on 1000 resamplings [23]. Sequences of *Taenia solium* from Kanchanaburi Province were used as outgroups (AB066487 for *cox1*, AB505027 for *ef1* and AB505025 for *elp*) to indicate the location of the root of the ingroup. For presentation purposes, the long branch leading to the outgroup is not shown for any tree.

Table 1
Primer pairs used for PCR.

Target genes	Primer names	Sequences (5′ – 3′)
cox1 (mt DNA)	Tsag_cox1/F	GAGGAAATTGTGAAGTTACTGCTA
	Tsag_cox1/R	ATGATGCAAAAGGCAAATAAACCT
ef1 (nuclear DNA)	Tae_ef1/F4	TGTGGTGGAATCGATAAAAGG
	Tae_ef1/R4	TCGATCTCATGTCACGAACG
elp (nuclear DNA)	Tsag_elp/F	CGTATGGAGAATGAACAGAAACTG
	Tsag_elp/R	CTGTGCATCGTGTTTCTCACGCAT

cox1: cytochrome *c* oxidase subunit 1.
ef1: elongation factor-1-alpha.
elp: ezrin/radixin/moesin-like protein.

3. Results

The mtDNA-based multiplex PCR tentatively assigned our samples to *T. asiatica* ($n=20$) or *T. saginata* ($n=18$). The phylogenetic tree inferred from complete mitochondrial *cox1* gene sequences (1620 bp) clearly identified two main and rather uniform clusters, agreeing with the results of the multiplex PCR (Fig. 1a). Although pairwise differences between "Tasi" and "Tsag" occurred at approximately 70 sites in the *cox1* gene, variations within each cluster were very small. In the case of the nuclear genes, introns were included in the fragments sequenced. The *ef1* gene fragment was 1095–1096 bp in length (60–61 bp introns and 1035 bp exons) and the *elp* gene sequences consisted of 1162–1164 bp (902–904 bp intron and 260 bp exons).

At the *ef1* locus, we found three alleles (*ef1A*, *ef1B* and *ef1C* — Fig. 1b). Two of these alleles were identified in "Tasi" (*ef1A*, *ef1B*), differing from each other at only a single site in an exon. Most of the samples were homozygous at this locus, but TasiA190 and TasiA210 were heterozygous. We found no variation in "Tsag" sequences (*ef1C*), with the exception of sample TsagA199, which was homozygous for an allele (*ef1A*) found at high frequency in "Tasi". The *ef1C* sequence differed from the other two alleles at 6–8 sites. Four alleles were found at the *elp* locus (*elpA*, *elpB*, *elpC* and *elpD* — Fig. 1c). The first two of these occurred in "Tasi" (*elpA*, *elpB*) and three in "Tsag" (*elpA*, *elpC*, *elpD*). Allele *elpA* thus occurred in both "Tasi" and "Tsag". Alleles *elpA* and *elpB* differed at 1–2 sites, as did *elpC* and *elpD*. Differences between these two pairs of alleles occurred at 6–7 sites. Samples TasiT010 and TsagA201 were heterozygous at this locus. Samples TsagA199 and TsagT017 were homozygous for *elpA*, the major allele found in "Tasi" (Fig. 1c).

For comparison, *ef1* and *elp* genes of *T. solium* both differ from their orthologs in *T. asiatica* and *T. saginata* at 47–50 sites (data not shown). This highlights the close relationship between the last two taxa. The mtDNA classification of all samples and their genotypes at the two nuclear loci are summarized in Table 2.

Nucleotide sequences from *Taenia* species in this study have been deposited into DDBJ/EMBL/GenBank databases under accession numbers AB465211–AB465248 for the *cox1* gene, AB462851–AB462890 for the *ef1* gene and AB462811–AB462850 for the *elp* gene.

4. Discussion

We examined the nucleotide sequences of one mitochondrial gene (*cox1*) and of alleles at two nuclear loci (*ef1* and *elp*) from taeniid worms, which had been tentatively identified as *T. asiatica* or *T. saginata* using multiplex PCR. Phylogenetic analyses of a mitochondrial gene and

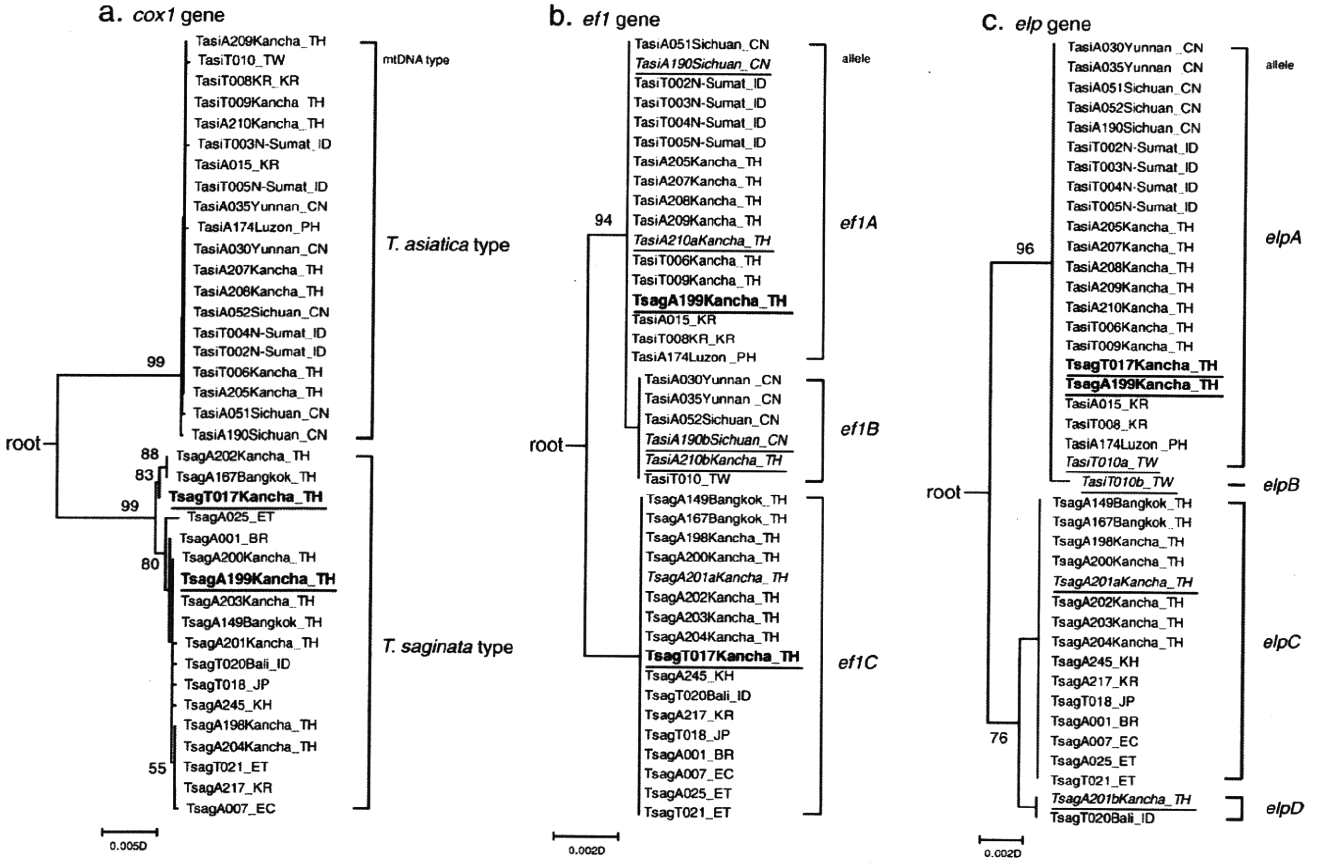

Fig. 1. Neighbor-joining phylogenetic trees of the mitochondrial cytochrome c oxidase subunit 1 gene (1a: *cox1*), nuclear genes for elongation factor-1-alpha (1b: *ef1*), and ezrin/radixin/moesin-like protein (1c: *elp*). Samples in italic type represent heterozygotes that displayed two alleles. Samples in bold type showed contradictions in the phylogeny between the mitochondrial gene and one or both of the nuclear genes. Numbers on the nodes represent bootstrap values. Each scale bar represents the evolutionary distances. The number after the species code (e.g. A030 or T002) identifies the sample ID used in the Asahikawa Medical College or Tottori University. Each sample code is followed by a locality name (absent from some) and country name (abbreviated). Abbreviations of country names are as follow: BR, Brazil; CN, China; EC, Ecuador; ET, Ethiopia; ID, Indonesia; JP, Japan; KH, Cambodia; KR, South Korea; PH, Philippines; TH, Thailand; TW, Taiwan. See the text for abbreviations of mitochondrial types and alleles.

two nuclear genes yielded trees consisting of two rather uniform clusters in each case. We regard the two clusters as corresponding to *T. asiatica* and *T. saginata*. The considerable difference between the mitochondrial lineages indicates a long period of separation between *T. asiatica* and *T. saginata*. Nuclear alleles *ef1A*, *ef1B*, *elpA* and *elpB* all occurred in *T. asiatica* even where it was not sympatric with *T. saginata*. We therefore regard these alleles as originating with that species. Similarly, *ef1C*, *elpC* and *elpD* were found in individuals of *T. saginata* from parts of the world where *T. asiatica* is not known. Regardless of the species identified, most individuals are homozygous at the nuclear loci. This is to be expected because taeniids are primarily self-fertilizers [24,25], a mating system that will lead to increase homozygosity. Some outcrossing has been demonstrated in *Echinococcus* species and should partially counter the trend towards increasing homozygosity [9]. The presence of a few heterozygous individuals in our study suggests that outcrossing also occurs in *Taenia* species. As shown in Table 2, these individuals are the samples TasiA210 and TasiA190 (heterozygous at *ef1*), TasiT010 (heterozygous at *elp*) and TsagA201 (heterozygous at *elp*).

Table 2
Samples used, their geographical origins and genotypes.[a]

Samples	mtDNA type	Genotype at *ef1* locus	Genotype at *elp* locus
14 samples[b]	*T. asiatica* type	*ef1A/ef1A*	*elpA/elpA*
3 samples[b]		*ef1B/ef1B*	*elpA/elpA*
TasiA190 Sichuan, China		*ef1A/ef1B*	*elpA/elpA*
TasiA210 Kanchanaburi, Thailand		*ef1A/ef1B*	*elpA/elpA*
TasiT010 Taiwan		*ef1B/ef1B*	*elpA/elpB*
14 samples[b]	*T. saginata* type	*ef1C/ef1C*	*elpC/elpC*
TsagT020 Bali, Indonesia		*ef1C/ef1C*	*elpD/elpD*
TsagA201 Kanchanaburi, Thailand		*ef1C/ef1C*	*elpC/elpD*
TsagT017 Kanchanaburi, Thailand		*ef1C/ef1C*	*elpA/elpA*
TsagA199 Kanchanaburi, Thailand		*ef1A/ef1A*	*elpA/elpA*

[a] See the text for abbreviations of mitochondrial haplotypes and alleles. The number after the species code (e.g. A190 or T010) identifies the sample ID used in the Asahikawa Medical College or Tottori University.
[b] See Fig. 1 for further details of geographical origins of samples.

Two individuals with mitochondrial genomes of the *T. saginata* type possessed at least some alleles typical of *T. asiatica*, suggesting a hybrid origin. TsagA199 has alleles typical of *T. asiatica* at both nuclear loci and TsagT017 displayed such allele at one locus (Table 2). Given the genotypes we observed in our other samples, neither of these individuals could have been an F1 hybrid because they were homozygous at both nuclear loci. Assuming descent from a hybrid ancestor, the observed genotypes could have arisen in two different ways. The presence of a mitochondrial genome of one species in the nuclear environment of another suggests mitochondrial introgression – past hybridization followed by backcrossing into the

paternal species and eventual dilution and loss of alleles inherited from the maternal species. It is possible that TsagA199 is a case of mitochondrial introgression. If so, additional nuclear loci in this individual should prove to be from *T. asiatica*. Alternatively, genotypes seen in both TsagA199 and TsagT017 could have arisen from a hybrid ancestor by selfing. The F1 hybrid would have been heterozygous at both loci. Its haploid gametes, however, following self-fertilization, could produce zygotes with the observed genotypes, each with a frequency of one-sixteenth in the next generation (F2). Further generations of selfing would eventually produce worms fixed at every locus for an allele from one parent species or the other. Testing between these two scenarios will simply require genotyping of additional nuclear loci in "pure" *T. saginata* and *T. asiatica* and in worms of supposed hybrid origin. The first scenario (mitochondrial introgression) requires many generations of backcrossing, unlikely in a taxon that predominantly self-fertilizes. The second scenario is therefore the more plausible.

In many animal taxa, hybrids are sterile. That is not the case for at least one of the worms studied here. When severe combined immunodeficiency (SCID) mice were infected with the eggs from TsagT017, mature cysticerci developed [26], demonstrating that the eggs had been fertilized and were viable. In addition, two of the cysticerci were homozygous at the *elp* locus and also at the cathepsin L-like cysteine peptidase locus, with each possessing the major allele found in *T. asiatica* [26]. In the second scenario above, the likelihood of a single egg from the F1 hybrid (F2) having such a genotype is also one-sixteenth. Thus, the hybrid-derived offspring observed in this study may not be from the F2 generation but from later generations.

Since hybrid-derived worms have been found only in Kanchanaburi Province, Thailand, where the two species are sympatrically endemic now, it is highly likely that hybridization between *T. asiatica* and *T. saginata* is an event in progress. However, it may not be very common: we observed hybrid-derived offspring in 13.3% of samples (2 cases/15 samples collected from Kanchanaburi). The difference of intermediate hosts utilized by *T. asiatica* and *T. saginata* may reduce the opportunity of a simultaneous infection with these two species. In fact, we have never found a mixed infection with *T. asiatica* and *T. saginata* in Kanchanaburi, although *T. asiatica* and *T. solium* were simultaneously found in humans because pig is the common intermediate host for both parasites [16].

Although we propose occasional crossing between *T. asiatica* and *T. saginata* as an explanation for our observations, it is also possible that ancestral polymorphism has been retained in what are actually two reproductively isolated species [9]. We argue that this is unlikely because self-fertilization by taeniid tapeworms will lead to rapid loss of some alleles and fixation of others. In addition, sequences of *ef1A* and *elpA* in "TsagA199" or "TsagT017" were identical with those in "Tasi", in introns as well as exons. Complete identity, especially in introns, would not be expected if there had been no gene exchange for long periods of time. Nevertheless, the possibility of retention of ancestral polymorphism cannot be completely dismissed by the present data.

When proglottids or eggs of taeniid cestodes are detected in human feces, the accurate identification of species is now dependent on specific PCR or the sequencing analysis of mtDNA [17,18,27]. The species of intermediate host is then inferred from the taeniid species identified. The occurrence of gene exchange between *T. saginata* and *T. asiatica* indicates that the intermediate host inferred from analysis of the mtDNA may not always be the correct one. In addition, the intermediate host cannot be identified from analysis of nuclear genes such as *ef1* and *elp* alone. Unless the gene or genes that regulate host specificity can be identified, the intermediate host should not be deduced based solely on DNA analysis in sympatric endemic areas. The inability to identify intermediate hosts accurately continues to be a significant epidemiological problem. It is necessary now to examine the genotypes of cysticerci from intermediate hosts (cattle and pigs) in sympatric endemic areas.

The results of this study strongly suggest that reproductive isolation is still incomplete between *T. saginata* and *T. asiatica*, and that hybrid breakdown does not occur. Although *T. asiatica* is still able to hybridize with *T. saginata*, each taxon has its own biological identity [12,15]. Biomedical researchers need more pragmatic approaches that can be understood by non-specialists [25]. At the very least, medical or veterinary researchers should not equate *T. asiatica* with *T. saginata*. Further population genetic studies are necessary to better understand the close relationship between these species. In particular, the finding of F1 hybrids is required to exclude the possibility of ancestral polymorphism.

Acknowledgements

This study was supported in part by Grant-in-Aid for Scientific Research from the Japan Society of Promotion of Science (JSPS) to M. Okamoto (18406008, 21406009) and to A. Ito (17256002, 21256003), by the Asia-Africa Science Platform Fund from JSPS to A. Ito (2006–2011) and by JSPS-Japan/China Bilateral Medical Joint Project on Taeniasis/Cysticercosis to M. Nakao (2009–2010). The authors thank the many colleagues and field workers who collected taeniid worms, especially Qiu DC, Li TY, Mamuti W, Eom KS, deLeon WU, Wandra T, Sutisna P, Depary AA, Benitez-Ortiz W, Nunes CM, Fan PC, Yoonuan T, Sato M, Tachi E and Suzuki Y.

References

[1] Xiao N, Qiu J, Nakao M, Li T, Yang W, Chen X, et al. *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika in China. Int J Parasitol 2005;35:693–701.
[2] Nakao M, McManus DP, Shantz PM, Craig PS, Ito A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitology 2007;134:713–22.
[3] Hüttner M, Nakao M, Wassermann, Siefert L, Boomker JDF, Dinkel A, et al. Genetic characterization and phylogenetic position of *Echinococcus felidis* Ortlepp, 1937 (Cestoda: Taeniidae) from the African lion. Int J Parasitol 2008;38:861–8.
[4] Thompson RCA, McManus DP. Towards a taxonomic revision of the genus *Echinococcus*. Trends Parasitol 2002;18:452–7.
[5] Thompson RCA. The taxonomy, phylogeny and transmission of *Echinococcus*. Exp Parasitol 2008;119:439–46.
[6] Saarma U, Jõgisalu I, Moks E, Varcasia A, Lavikainen A, Oksanen A, et al. A novel phylogeny for the genus *Echinococcus*, based on nuclear data, challenges relationships based on mitochondrial evidence. Parasitology 2009;136:317–28.
[7] Lavikainen A, Haukisalmi V, Lehtinen MJ, Henttonen H, Oksanen A, Meri A. A phylogeny of members of the family Taeniidae based on the mitochondrial *cox1* and *nad1* gene data. Parasitology 2008;135:1457–67.
[8] Mayr E. What is a species, and what is not? Philos Sci 1996;63:262–77.
[9] Badaracco JL, Ayala FJ, Bart JM, Gottstein B, Haag KL. Using mitochondrial and nuclear markers to evaluate the degree of genetic cohesion among *Echinococcus* populations. Exp Parasitol 2008;119:453–9.
[10] Haag KL, Gottstein B, Ayala FJ. The EG95 antigen of *Echinococcus* spp. contains positively selected amino acids, which may influence host specificity and vaccine efficacy. PLoS ONE 2009;4:e5362.
[11] Ito A, Nakao M, Wandra T. Human taeniasis and cysticercosis in Asia. Lancet 2003;362:1918–20.
[12] Eom KS. What is Asian *Taenia*? Parasitol Int 2006;55 suple:S137–41.
[13] McManus DP. *Taenia* in the gastrointestinal tract. N Engl J Med 2008;358:311.
[14] Bowles J, McManus DP. Genetic characterization of the Asian *Taenia*, a newly described taeniid cestode of humans. Am J Trop Med Hyg 1994;50:33–44.
[15] Hoberg EP. Phylogeny of *Taenia*: Species definitions and origins of human parasites. Parasitol Int 2006;55 suple:S23–30.
[16] Anantaphruti MT, Yamasaki H, Nakao M, Waikagul J, Watthanakulpanich D, Nuamtanon S, et al. Sympatric occurrence of *Taenia solium*, *T. saginata*, and *T. asiatica*, Thailand. Emerg Infect Dis 2007;13:1413–6.
[17] Yamasaki H, Allan JC, Sato MO, Nakao M, Sako Y, Nakaya K, et al. DNA differential diagnosis of taeniasis and cysticercosis by multiplex PCR. J Clin Microbiol 2004;42:548–53.
[18] Jeon HK, Chai JY, Kong Y, Waikagul J, Insisiengmay B, Rim HJ, et al. Differential diagnosis of *Taenia asiatica* using multiplex PCR. Exp Parasitol 2009;121:151–6.
[19] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
[20] Saitou N, Nei M. The neighbor-joining method: a new method for reconstruction phylogenetic trees. Mol Biol Evol 1987;4:406–25.
[21] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.

[22] Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. PNAS 2004;101:11030–5.
[23] Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39:783–91.
[24] De Meeus T, Durand P, Renaud F. Species concept: what for? Trend Parasitol 2003;19:425–7.
[25] Tibayrenc M. The species concept in parasites and other pathogens: a pragmatic approach? Trend Parasitol 2006;22:66–70.
[26] Nkouawa A, Sako Y, Nakao M, Nakaya K, Ito A. Loop-mediated isothermal amplification method for differentiation and rapid detection of *Taenia* Species. J Clin Microbiol 2009;47:168–74.
[27] Eom KS, Jeon HK, Kong Y, Hwang UW, Li X, Xu L, et al. Identification of *Taenia asiatica* in China: molecular, morphological, and epidemiological analysis of a Luzhai isolate. J Parasitol 2002;88:758–64.

Parasitology International 59 (2010) 326–330

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Molecular and serological survey on taeniasis and cysticercosis in Kanchanaburi Province, Thailand

Malinee T. Anantaphruti [a,b,*,1], Munehiro Okamoto [c,1], Tippayarat Yoonuan [a], Surapol Saguankiat [a], Teera Kusolsuk [a], Megumi Sato [a], Marcello O. Sato [b], Yasuhito Sako [b], Jitra Waikagul [a], Akira Ito [b,1]

[a] Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand
[b] Department of Parasitology, Asahikawa Medical College, Asahikawa 078-8510, Japan
[c] Department of Parasitology, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

ARTICLE INFO

Article history:
Received 9 February 2010
Received in revised form 17 March 2010
Accepted 18 March 2010
Available online 7 April 2010

Keywords:
Taeniasis
Cysticercosis
Molecular diagnosis
ELISA
Immunoblot
Kanchanaburi, Thailand

ABSTRACT

A community-based field survey on taeniasis and cysticercosis was performed in two villages in Thong Pha Phum District, Kanchanaburi Province, central Thailand, where 3 *Taenia* species, *T. solium*, *T. saginata* and *T. asiatica*, are sympatrically occurring. Four (0.6%) out of 667 stool samples were egg-positive for *Taenia* sp. by Kato–Katz technique. Three out of those four persons and other three persons who were *Taenia* egg-negative but having a recent (<1 year) history of discharging worms in stool were treated with niclosamide. One *Taenia* egg-positive woman was not treated because of severe ascites. After treatment, three persons expelled long strobilae with scolices and two persons expelled strobilae without scolex. One *Taenia* egg-positive person did not expel any worms post-treatment. Among 5 persons, four expelled a single worm, whereas one expelled multiple worms, may be 6 worms but not confirmed by detection of scolices. One scolex was armed with hooklets, whereas 2 others did not. Multiplex PCR of 10 expelled proglottids (including 6 estimated worms from one patient) revealed that one sample was *T. solium*, one *T. saginata*, and 8 *T. asiatica*. A total of 159 residents agreed to receive a serological test for cysticercosis. By ELISA using partially purified glycoprotein antigen, 9 cases, 5 and 4 from villages A and B respectively, were found to be sero-positive. The five and an additional sample on the border line from village A were evaluated using confirmative immunoblot using recombinant chimeric antigen. Among the six samples, four including the border line sample were confirmed to be cysticercosis by immunoblotting. One of the 4 persons had neurological symptoms with nodular lesions in the brain by computed tomography. These 4 confirmed or suspected cysticercosis cases were free of *T. solium* worms, but two of them including confirmed NCC case had a past (>1 year) history of expelling proglottids in the stool.

1. Introduction

A nationwide stool survey in Thailand by the Ministry of Public Health revealed 1–2% of taeniasis infection in the north and northeast of the country, with <1% in the central region, and almost none in the south [1]. It has been reported that human cases of *Taenia saginata* are rather common [2] but those of *Taenia solium* are few [3]. However, cysticercosis cases have often been found in hospital records [4,5]. Until 2007, there was no evidence of the occurrence of *Taenia asiatica* in Thailand. Through the field work from 2002 until 2005 in Thong Pha Phum District, Kanchanaburi Province, Thailand, close to the Myanmar border, both *T. solium* and *T. saginata* were found in the same area. By using molecular approaches [6], the morphologically identified *T. saginata* in this area included not only *T. saginata* but also *T. asiatica* and, therefore, three *Taenia* species, *T. asiatica*, *T. saginata* and *T. solium* sympatrically occurred there [7].

As cysticercosis of *T. solium* is one of the most lethal parasitic diseases and difficult to prevent the spread of it in the community [8,9], we were keen to control cysticercosis at the community basis. Since three *Taenia* species were found to be endemic in this area, Kanchanaburi Province [7], we applied serological screening of cysticercosis and molecular identification of *Taenia* worms from patients to draw precise epidemiology of taeniasis and cysticercosis in this area.

2. Materials and methods

2.1. Study community

The study area was Thong Pha Phum District, 150 km northwest of Kanchanaburi, a province in central Thailand, 130 km west of Bangkok.

* Corresponding author. Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchathewee, Bangkok 10400, Thailand. Tel./fax: +66 2 643 5600.
E-mail address: tmmtr@mahidol.ac.th (M.T. Anantaphruti).
[1] These authors equally contributed for the preparation of the paper.

1383-5769/$ - see front matter © 2010 Published by Elsevier Ireland Ltd.
doi:10.1016/j.parint.2010.03.007