Table 3
Details of PCR positive results.

Parasite	Host	Course	Sample	PCR		Comments
				Primers	Result (bp)	
S. japonicum	Mice	1 DPI	Pooled urine	CF/CR	254	
		3 DPI	Pooled urine	CF/CR	254	
		1 WPI	Pooled urine	CF/CR	254	
		2 WPI	Pooled urine	CF/CR	254	
		3 WPI	Pooled urine	CF/CR	254	
		4 WPI	Pooled urine	CF/CR	254	
		5 WPI	Pooled urine	CF/CR	254	
	Mice	1 WPI	Pooled urine	SjF/CR	614	
		2 WPI	Pooled urine	SjF/CR	614	
S. mansoni	Mice	1 DPI	Pooled urine	CF/CR	254	
		3 DPI	Pooled urine	CF/CR	254	
		1 WPI	Pooled urine	CF/CR	254	
		2 WPI	Pooled urine	CF/CR	254	
		3 WPI	Pooled urine	CF/CR	254	
		4 WPI	Pooled urine	CF/CR	254	
		5 WPI	Pooled urine	CF/CR	254	
	Mice	4 DPI	Pooled urine	SmF/CR	479	
		5 DPI	Pooled urine	SmF/CR	479	
S. haematobium	Gerbil	34 WPI	Serum	ShF/CR	365	Lesion(−), unisexual
	Gerbil	36 WPI	Serum	ShF/CR	365	Lesion(−), unisexual
	Hamster	~1 year	Serum	ShF/CR	365	Lesion(+)/worm(+)
S. mekongi	Mouse	1 DPI	Serum, urine*	SmekF/CR	303	*2nd PCR
		1 WPI	Serum, urine	SmekF/CR	303	
		2 WPI	Urine	SmekF/CR	303	
		3 WPI	Serum, urine	SmekF/CR	303	
		4 WPI	Serum*, urine	SmekF/CR	303	*2nd PCR
		5 WPI	Serum, urine	SmekF/CR	303	
		6 WPI	Serum	SmekF/CR	303	
	Mouse	3 DPI	Serum	SmekF/CR	303	*2nd PCR
	Mouse	5 DPI	Serum	SmekF/CR	303	
	Mouse	4 WPI	Serum	SmekF/CR	303	
	Mouse	7 WPI	Serum	SmekF/CR	303	
	Mouse	8 WPI	Serum	SmekF/CR	303	
	Mouse	8 WPI	Serum	SmekF/CR	303	
	Dog	>3 years	Serum, urine	SmekF/CR	303	No eggs in feces
	Dog	>3 years	Urine	SmekF/CR	303	No eggs in feces
	Hamster	19 WPI	Serum	SmekF/CR	303	Lesion(+)/worm(+)
	Gerbil	17 WPI	Serum	SmekF/CR	303	Lesion(+)/worm(+)

DPI: days-post-infection.
WPI: weeks-post-infection.
* 2nd PCR: positive result was obtained by the 2nd PCR.

those who have hepatic lesions but no viable worms or eggs, the administration of praziquantel is not required, however, such patients do need supportive measures. As the result, further details about circulating schistosome DNA in the host therefore need to be elucidated.

We have developed simple PCR systems that can differentiate four human schistosome species (*S. mansoni*, *S. haematobium*, *S. japonicum* and *S. mekongi*). PCR can be successfully performed by combining primers for the different species and/or that for a schistosome common region. The use of common reverse primer (CR) allows for simple handling and thus makes this diagnostic modality suitable for performing accurate species differentiation. From our accumulative data obtained using experimental animals, parasite DNA was detected from 1 DPI at the earliest (Table 3). We are presently assessing this usefulness of this PCR diagnostic modality for samples obtained from patients in schistosomiasis endemic areas. The PCR method described herein is therefore considered to be a potentially useful diagnostic tool for human schistosomiasis, independent of the presence of parasite eggs.

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Short Report: Protein Kinase A Regulatory Subunit Interacts with P-Type ATPases in *Trypanosoma cruzi*

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Abstract. Cyclic AMP–protein kinase A (PKA) signaling is important for the growth and differentiation of *Trypanosoma cruzi*. Immunofluorescence suggests that PKA can associate with the plasma membrane of trypomastigotes. We found that the PKA regulatory subunit interacts with several P-type ATPases. These P-type ATPases may play a role in anchoring PKA to the plasma membrane in *T. cruzi*.

Trypanosoma cruzi is the etiologic agent of Chagas disease and is also seen as an opportunistic infection in immune-compromised patients.[1] This organism has four distinct developmental life stages: the epimastigote and metacyclic trypomastigote in its insect vector, the amastigote, an intracellular form, and trypomastigote, a blood form, in its mammalian host. Cyclic AMP-protein kinase A (PKA) signaling has been implicated as a regulator of stage differentiation in *T. cruzi*.[2,3] In mammalian cells, the molecular explanation for the specificity of PKA is provided by its compartmentalization through association with A-kinase–anchoring proteins.[4] In *T. cruzi*, the mechanism for PKA targeting remains unknown. Previously, we reported on our characterization of both the catalytic (TcPKAc) and regulatory (TcPKAr) subunits of PKA in *T. cruzi*.[5,6] To identify potential anchoring proteins of TcPKAr, we performed yeast two-hybrid and co-immunoprecipitation assays and found that several P-type ATPases interacted with TcPKAr.

P-type ATPases are membrane proteins that can transport specific ions, including Na^+, K^+, Ca^{2+}, and H^+, across membranes against concentration gradients. We previously reported the molecular cloning and characterization of a *T. cruzi* Na^+-ATPase that plays a role in adaptation of different ionic environments.[7] In mammalian cells, a P-type ATPase, the Na^+-K^+-ATPase, has been reported to anchor a group of proteins mediating signal transduction activating multiple protein kinase cascades including mitogen-activated protein kinase and protein kinase C.[8] Our data suggest that P-type ATPases may have a similar function as anchoring proteins for PKA in *T. cruzi*.

Trypanosoma cruzi cell culture techniques and immunofluorescence analysis (IFA) using monoclonal antibody (mAb) such as anti-PKAr have been previously described by our laboratory group.[5,6] To identify PKAr anchoring proteins, a large-scale yeast two-hybrid screen was performed using the techniques we previously described.[9] Briefly, a bait construct (using BD the binding domain of GAL4) was produced by ligating the full-length open reading frame (ORF) of TcPKAr[6] (GenBank accession number AF532200) into pBD plasmids at *Sal*1 to generate pBD-TcPKAr. Large-scale transformation of the bait construct pBD-TcPKAr with a *T. cruzi* AD plasmid library was carried out using YRG-2 yeast competent cells under a high stringency screen according to the manufacturer's protocol (Stratagene, La Jolla, CA). Information from prey gene sequences was analyzed using BLAST (GenBank, NCI).

Entire ORFs of these prey genes or partial genes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from CL Brener strain epimastigotes as described in our previous publication,[9] with the following primers containing appropriate restriction sites: partial cation-transporting ATPase, (Tc00.1047053509611.80; C-terminal 329 amino acids); forward (*Xho*I) 5′-CCGCTCGAGGCGGATGCCCCGTTTATGGT-3′, reverse (*Pst*1) 5′-TTCTGCAGTCACATGTAGTCCGTCAGA-3′; full-length Na^+-ATPase (GenBank accession number AB107891), forward (*Bam*H1) 5′-CGGGATCCATGTCGGATTTGAAAGAGCTAAGCAT-3′; reverse (*Xho*I) 5′-CCGCTCGAGCTAACGCCTCTTCTTTTTCCTCTC-3′; full-length calcium motive P-type ATPase, putative (Tc00.1047053510769.120) forward (*Bam*H1) 5′-CGGGATCCATGTCGGATTCGAAAGAGCTAAGCA-3′; reverse (*Xho*I) 5′-CCGCTCGAGCTAACGCCTCTTCTTTTTCCTCTC-3′. Briefly, cDNA was generated using the SuperScript First-Strand Synthesis System for RT-PCR according to manufacturer's protocol (Invitrogen, Carlsbad, CA). cDNA was used as a template to amplify genes by PCR. Amplicons were ligated into pAD-Gal4 vectors and re-transformaed using pBD- TcPKAr and pAD-prey genes under the highest stringency conditions (-Leu, -Trp, -His).[9] Positive control (pADwt with pBDwt) and negative control (pADwt with pLaminC) reactions provided by the manufacturer (Invitrogen) were included as a quality control.

The generation of a specific antibody for Na^+-ATPase was previously described by Iizumi and others.[7] A new antiserum in Balb/c mice was produced to recombinant protein from a 200 amino acid region of the C terminus of *T. cruzi* P-type ATPase (Tc00.1047053509611.80) using Freund's adjuvant (Sigma, St. Louis, MO) at a 1:1 dilution with boosting at 4 and 12 weeks. The specificity of this C-terminal antiserum was confirmed by immunoblot.

Bi-directional co-immunoprecipitations were performed to confirm protein–protein interactions as described previously.[9] Briefly, a Triton X-100 extract from trypomastigotes was incubated with TcPKAr mAb, antibodies of *T. cruzi* P-type antibodies (1:100) at 4°C overnight, and the immunocomplex was precipitated with protein A-Sepharose CL-4B (Sigma), washed, resuspended in 30 μL sample buffer, boiled for 5 minutes, centrifuged, run on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and blocked with 5% non-fat milk. TcPKAr mAb immuno-complex was detected with *T. cruzi* P-type ATPase antibodies (1:1,000 dilution), whereas immuno-complexes pulled down by P-type ATPase antibodies were detected with TcPKAr mAb (1:1,000 dilution) and secondary anti-native mouse IgG antibody con-

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jugated with horseradish peroxidase (1:5,000 dilution; Pierce Biotechnology, Rockford, IL) and visualized using a chemiluminescent substrate.

IFA using mAbs against TcPKAr showed that, in trypomastigotes, TcPKAr was associated with the flagellum and membrane. In addition, subcellular fractionation followed by immunoblot analysis indicated that TcPKAr was present in the membrane-associated fraction in trypomastigotes (Supplemental Figure). In addition to this membrane staining, cytoplasmic staining was also evident in all three forms of *T. cruzi*, and secondary antibody did not show staining (data not shown). Under high stringent selection conditions (-Leu, -Trp, -His),[9] two full-length and a C-terminal partial P-type ATPase were confirmed to interact with TcPKAr (Figure 1); these proteins were as follows: 1) a cation-transporting ATPase, putative (Tc00.1047053509611.80; C-terminal 329 amino acids); 2) a calcium motive P-type ATPase, putative (Tc00.1047053510769.120; full length); and 3) a Na^+-ATPase (GenBank accession number AB107891; full length).[7] Immuno-complexes precipitated by TcPKAr mAb contained an 120-kDa band, which was recognized by antibodies to Na^+-ATPase, and an 140-kDa band that was recognized by anti-cation-transporting ATPase, indicating that TcPKAr associated with P-type ATPases. Complexes from both anti-Na^+-ATPase and anti-cation-transporting ATPase contained a 56-kDa band recognized by TcPKAr mAb (Figure 2).

PKA is held in an inactive form by the association of its two catalytic subunits (PKAc) with a PKAr dimer. When cAMP concentration increases, PKAr binds cAMP at two sites and dissociates from PKAc, allowing PKAc to phosphorylate its substrates.[10] In metazoans, PKAr binds to A-kinase anchoring proteins with high affinity, mediating the compartmentalization of PKA and ensuring its specificity by placing PKAc close to its appropriate effectors and substrates.[4] In *T. cruzi*, the PKAr binding proteins remain unknown. We identified several P-type ATPases that interact with PKAr. We also tested whether TcPKAc would interact with these P-type ATPases in the yeast two-hybrid system and found that they could not interact in this system (data not shown). This indicates that interactions of TcPKAr and these P-type ATPases are specific. One of the candidates turned out to be a Na^+-ATPase mediating adaptation for high Na^+, which we had reported previously.[7] These are the first PKAr binding proteins identified in *T. cruzi*.

P-type ATPases are expressed in all living organisms.[11] The primary structures of various P-type ATPase catalytic subunits contain eight conserved motifs solvated in the cytoplasm, where ATP binding and hydrolysis occur.[11,12] The primary function of Na^+-K^+-ATPase is to transport ions across the cell membrane. However, some mammalian Na^+-K^+-ATPase α-subunits contains multiple well-characterized protein-binding motifs, all of which are located outside of the eight conserved core regions of P-type ATPases.[11] In humans Na^+-K^+-ATPase serves as an anchor for a signalosome.[8]

TcPKAr can be seen on the plasma membrane of trypomastigotes and co-precipitates with P-type ATPases. Na^+-ATPase expression is increased in trypomastigotes.[7] Such P-type ATPases probably play a role in anchoring TcPKAr to the membrane. The trypomastigote is the infective form, which encounters both host immune responses and ionic alterations as it is released from host cells. Anchoring TcPKAr to the membrane may bring the holoenzyme to the vicinity of parasite surface, where it could alter the phosphorylation of key proteins involved in invasion and/or adaptation to changes in the environment. A hydropathy plot of Na^+-ATPase showed multiple cytoplasmic domains, including an N-terminal domain, a small loop, a large loop, and a C-terminal domain.[7] Alignments of the amino acid sequence showed that conserved sequences among P-type ATPases exist in the cytoplasmic domains of this protein.[7] Further studies of the motifs that mediate interaction between TcPKAr and these P-type ATPase proteins may lead to the understanding of the mechanism of TcPKAr targeting to the membrane.

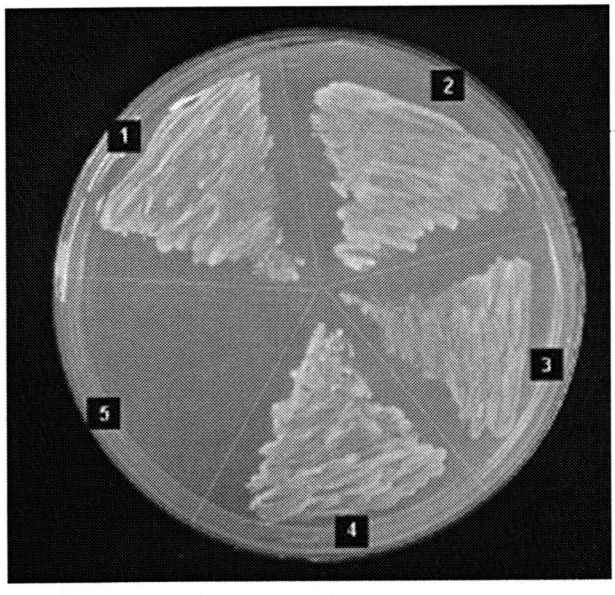

FIGURE 1. P-type ATPases interact with TcPKAr. Yeast two-hybrid screen (high stringency selection) using two full-length and one C-terminal construct of P-type ATPases from *T. cruzi* in pAD-Gal4 vectors showing an interaction with the pBD-TcPKAr vector. **1**, Cation-transporting ATPase, putative (Tc00.1047053509611.80), C-terminal 329 amino acids. **2**, Calcium motive P-type ATPase, putative (Tc00. 1047053510769.120), full length. **3**, Na^+-ATPase (GenBank accession number AB107891), full length.[7] **4**, Positive control (pADwt with pBDwt). **5**, Negative control (pADwt with pLaminC). This figure appears in color at www.ajtmh.org.

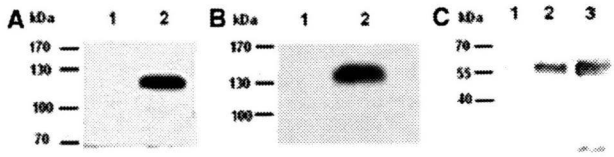

FIGURE 2. Immunoprecipitation of TcPKAr and P-type ATPases. Immune complexes were pulled down by TcPKAr mAb, anti-cation-transporting ATPase, and anti-Na^+-ATPase. The components of the complexes were detected by immunoblot using appropriate antibodies. No pull down of P type ATPases occurred with anti-BAG5 and pre-immune antisera, but pull down was clearly evident with anti-TcPKAr mAb, anti-cation-transporting ATPase, and anti-Na^+-ATPase. **A**, Lane 1 is a negative control using an unrelated antiserum (Anti-BAG5 mAb, which reacts with a small heat shock protein of *Toxoplasma gondii*). Lane 2 used anti-TcPKAr mAb for the immunoprecipitation. In both lanes, anti-Na^+-ATPase was used for the immunoblot.[7] **B**, Lanes 1 and 2 are the same as **A**. In both lanes, anti-cation-transporting ATPase was used for the immunoblot. **C**, Lane 1 is a negative control using pre-immune mouse serum. Lane 2 uses anti-Na^+-ATPase immunoprecipitation. Lane 3 uses anti-cation-transporting ATPase immunoprecipitation. TcPKAr mAb was used for the immunoblot of all three lanes.

Note: A supplemental figure (Immunofluorescence analysis of *T. cruzi* using anti-TcPKAr [MAb] and TcPKAr is in membrane-associated fraction in trypomastigote) appears online at www.ajtmh.org.

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Specific IgG Responses to Recombinant Antigen B and Em18 in Cystic and Alveolar Echinococcosis in China[▽]

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An understanding the correlation of the specific antibody responses and the disease phase is essential in evaluating diagnostic values of immunological tests in human echinococcosis. In this study, 422 echinococcosis patients diagnosed by ultrasonography, including 246 with cystic echinococcosis (CE), 173 with alveolar echinococcosis (AE), and 3 with dual infection, were tested for specific IgG in sera against recombinant AgB (rAgB) and recombinant Em18 (rEm18) in an enzyme-linked immunosorbent assay. As a result, rAgB-specific antibody was detected in 77.6% of CE and 86.1% of AE patients, while rEm18-specific antibody was present in 28.9% of CE and 87.3% of AE patients. Additionally, all three patients with dual infection exhibited specific antibodies responding to rAgB and rEm18. Further analysis revealed that rAgB-specific antibody was elevated in a significantly greater proportion (87.3%) of CE patients with cysts at active or transitional stages (CE1, CE2, or CE3), compared to 54.8% of other patients with cysts at an early or an inactive stage (CL or CE4 or CE5). Furthermore, rAgB-specific antibody was detected in 95.6% of CE2 cases, which was statically greater than that (73.7%) in CE1 patients. Although rEm18-specific antibody was elevated in 28.9% of CE patients, the positive reaction was much weaker in CE than in AE cases. Serum levels and concentrations of rEm18-specific antibody were further indicated to be strongly disease phase correlated in AE patients, with positive rates of 97.4% in cases with alveolar lesions containing central necrosis and 66.7% in patients with early alveolar lesions that measured ≤5 cm.

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Humans acquire the infection of echinococcosis by accidental ingestion of eggs excreted with feces of carnivores harboring the adult worms of *Echinococcus* spp. The eggs hatch in the small intestine of humans, releasing the oncosphere, which migrates via the portal system into various organs and then develops into the metacestode stage. The larval parasite can establish itself in any part of the human body but most frequently does so in the liver (32). Diagnosis of human echinococcosis is primarily based on the pathognomonic features in images obtained using imaging techniques including ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI). Of these techniques, B-ultrasound is much more widely applied, as CT and MRI are too expensive and largely inaccessible in most areas where echinococcosis is endemic. Criteria for classification of cystic echinococcosis (CE) and alveolar echinococcosis (AE) have been proposed based on stage-specific ultrasound images (21, 36). Briefly, on the basis of conformational features of cysts, CE lesions are differentiated into six types: CL, CE1, CE2, CE3, CE4, and CE5. The CL type refers to a cystic lesion of a parasite origin and without a clear rim, indicating the parasite is at a very early stage of development. The CE1 type describes a unilocular simple cyst with uniform anechoic content, and importantly, with a visible wall, while the CE2 type is characterized by multivesicular, multiseptated cysts in which daughter cysts may partially or completely fill the unilocular mother cyst. The presence of CE1 or CE2 cysts is indicative of an active stage of the disease. The CE3 type is distinguished by detachment of the cyst membrane and/or partial degeneration of cyst content, suggestive of a transitional parasite. A CE4 or CE5 type of cyst shows an involution, with a necrotic or inactive parasite, with the features of complete degeneration of cyst content for CE4 and a calcified cyst wall for CE5 (36). In contrast, AE lesions are characterized by a nonhomogenous hyperechoic tumor-like structure with a poorly defined verge and containing scattered calcifications and/or a central necrotic cavity (1), and they are further differentiated into three types and eight subtypes based on the features and sizes of lesions, including AE1, AE2, and AE3 (21). In detail, AE1 refers to alveolar lesions measuring ≤5 cm, normally without central necrosis detected, and the type is differentiated further as AE1s (single lesion) and AE1m (multiple lesion) subtypes and indicates an early stage of the disease. Alveolar lesions that measure >5 cm and ≤10 cm are classified as AE2 and include three subtypes, recorded as AE2s (single lesion), AE2m (multiple lesions), and AE2f (presence of central necrotic fluid, regardless of the number of lesions), suggestive of a developing parasite, while AE lesions that measure >10 cm in diameter are confirmed as AE3, indicative of an advanced stage of the disease; this type

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includes three subtypes, i.e., AE3s (single lesion), AE3m (multiple lesions), and AE3f (presence of central necrotic fluid).

Meanwhile, several antigens, such as antigen B (AgB) (15, 20, 24, 26) for cystic echinococcosis and for *Echinococcus multilocularis* Em2a (8), II/3 (34), II/3-10 (27), EM10 (5), EM4 (9), and Em18 (12, 30), have been confirmed to be of potential use in serodiagnosis of human echinococcosis. However, relatively little information about the correlation between the specific antibody levels in humans and disease pathology or stage is available (29).

In this study, serum levels and concentrations of specific IgG antibodies in human CE and AE patients at different stages were determined by enzyme-linked immunosorbent assay (ELISA) using recombinant antigen B (rAgB) and recombinant Em18 (rEm18) as antigens.

MATERIALS AND METHODS

AQ: B **Serum samples.** A total of 422 serum samples were collected from 422 individuals with confirmative ultrasound images of echinococcal lesions during 2001 to 2008 in Tibetan communities of northwest Sichuan (23). We also performed all ultrasound examinations. Of these 422 individuals, 246 were diagnosed as CE, 173 as AE, and 3 as dual infection with both CE and AE. According to the criteria for classification of ultrasound images of cystic echinococcosis (36), 5 of the 246 CE cases were determined to have CL cysts of a parasitic origin (CL cysts of nonparasite origin were excluded in this study), 57 had CE1-type cysts, 68 had cysts belonging to the CE2 type, 39 had CE3 cysts, and 68 had CE4 or CE5 cysts. Two or more cystic lesions belonging to different types were concurrently observed in nine additional cases. Of 173 AE cases, 21 were classified as AE1, 54 as AE2 (without necrotic cavity), 20 as AE3 (without necrosis), and an additional 78 were grouped as AEf, including AE2f and AE3f. Serum samples were stored at −20°C until tested.

rAgB and rEm18 ELISAs. The rAgB and rEm18 antigens were prepared as described previously (26, 30). Each serum sample was analyzed in an ELISA for specific IgG antibody responses to rAgB and rEm18 as reported previously (26, 30), with a minor alteration. In the assays, a 100-μl volume was applied throughout unless otherwise stated and phosphate-buffered saline (PBS) containing 0.05% Tween 20 was employed as the washing buffer (PBST), while casein buffer (1% casein in 20 mM Tris-HCl [pH 7.6] containing 150 mM NaCl) was used as diluting solution of serum and conjugate and also as blocking solution. PBS was employed to dilute antigens. Briefly, 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with diluted antigen at a protein concentration of 0.5 μg/ml for rAgB and 1.0 μg/ml for rEm18 and incubated at 4°C overnight. After wells were rinsed three times with PBST, 300 μl of blocking solution was added to each well. Plates were incubated at 37°C for 1 h and washed five times. Serum samples diluted at 1:100 were added in duplicate wells and incubated at 37°C for 1 h. After washing five times, plates were incubated with rec-protein G-peroxidase conjugate (Invitrogen, Camarillo, CA) at a 1:4,000 dilution at 37°C for 1 h. Plates were washed five times and incubated with substrate solution [0.4 mM 2,2′-azino-bis(3-ethybenzthiazoline-6-sulfonic acid) (ABTS) in 0.1 M citric acid buffer and 0.2 M Na_2HPO_4] at room temperature for 30 min. The color reaction was stopped by application of 1% SDS in each well. The optical density (OD) was determined at 405 nm with a microplate ELISA reader (model 450; Bio-Rad Laboratories, Hercules, CA).

The cutoff points were determined as the mean optical density of 30 serum samples obtained from healthy donors plus 3 standard deviations (SD).

Statistical analyses. A chi-square test was used for comparing sensitivities among patients grouped on the basis of the type of echinococcal lesion, and the Kruskal-Wallis H rank sum test was applied to compare ELISA OD values for groups of patients with lesions at different stages, whereas the Wilcoxon rank AQ: C sum test was used to compare OD values between groups of patients. *P* values equal to or less than 0.05 were considered indicative of statistical significance.

RESULTS

The cutoff values (mean OD plus 3 SD) derived from analysis of negative control sera (n = 30) were 0.048 for rAgB and 0.076 for rEm18.

TABLE 1. Results of ELISAs with rAgB and rEm18 as antigens in 246 CE patients

Cyst type(s)	No. of patients examined	No. (%) of patients with positive response to:	
		rAgB	rEm18
CL	5	2 (40.0)	0 (0)
CE1	57	42 (73.7)	16 (28.1)
CE2	68	65 (95.6)	31 (45.6)
CE3	39	35 (89.7)	14 (35.9)
CE4/CE5[a]	68	38 (55.9)	7 (10.3)
Mixed	9	9 (100.0)	3 (33.3)
Total	246	191 (77.6)	71 (28.9)

[a] The patients with CE4 or CE5 cysts (indicative of inactive parasites) were grouped together.

CE. (i) rAgB ELISA. Of the 246 CE cases, a total of 77.6% (191) showed a positive IgG antibody response to rAgB, and the patients with positive reactions had a median OD of 0.640. However, patients with CL or CE4/CE5 cysts exhibited lower activities than those with CE1, CE2, or CE3 cysts. That is, 2 of 5 patients with CL cysts and 55.9% (38/68) of persons with CE4/CE5 cysts responded to rAgB, whereas specific antibody was detected in 73.7% (42/57) of CE1 cases, 95.6% (65/68) of CE2 cases, 89.7% (35/39) of CE3 cases, and in all 9 patients with mixed types of cysts (Table 1). Further analysis revealed T1 that antibody activity against rAgB was significantly different between CE patients with cysts at the early CL or inactive CE4/CE5 stage (40/73; 54.8%) and patients with active or transitional cysts (CE1, CE2, or CE3; 151/173; 87.3%) (χ^2 = 31.09; P = 0.000). Moreover, OD values in patients with active or transitional cysts were greater than those in patients with early or inactive cysts (P = 0.000) (Fig. 1A). Additionally, CE1 F1 patients had a significantly lower positive rate (73.7%) than CE2 patients (95.6%; χ^2 = 11.97; P = 0.005) (Table 1), and the difference in mean OD values was also significant (P = 0.000) (Fig. 1A).

(ii) rEm18 ELISA. Sera from CE patients were also tested using rEm18 as antigen. Of the 246 CE cases, 28.9% (71) showed a positive response to rEm18, and all the cases with seropositivity had an OD median of 0.135. Antibody levels against rEm18 varied among patients with different types of cysts. That is, all 5 patients with CL cysts showed a negative response, while specific antibody was detected in 45.6% (31/68) of CE2 patients, 35.9% (14/39) of CE3 cases, 3 of 9 CE cases with mixed types of cysts, 28.1% (16/57) of CE1 patients, and 10.3% (7/68) of CE4/CE5 cases (Table 1). However, reactions with rEm18 in CE patients were generally weak, with respective OD medians for each group (in parentheses), as follows: CL (0.008), CE1 (0.021), CE2 (0.063), mixed (0.051), CE3 (0.052), and CE4/5 (0.027) (Fig. 1C). The difference was significant (P = 0.000).

Of the 71 CE cases with a positive response to rEm18, all except 4 also exhibited specific antibody to rAgB at a rather high level (median, 0.994) (Fig. 1E). The four negative sera consisted of CE1 (two), CE3 (one), and CE4/CE5 (one).

AE. (i) rAgB ELISA. In 173 AE patients, 149 (86.1%) contained protein G binding antibodies (IgG) that recognized rAgB from *Echinococcus granulosus*, with an OD median of

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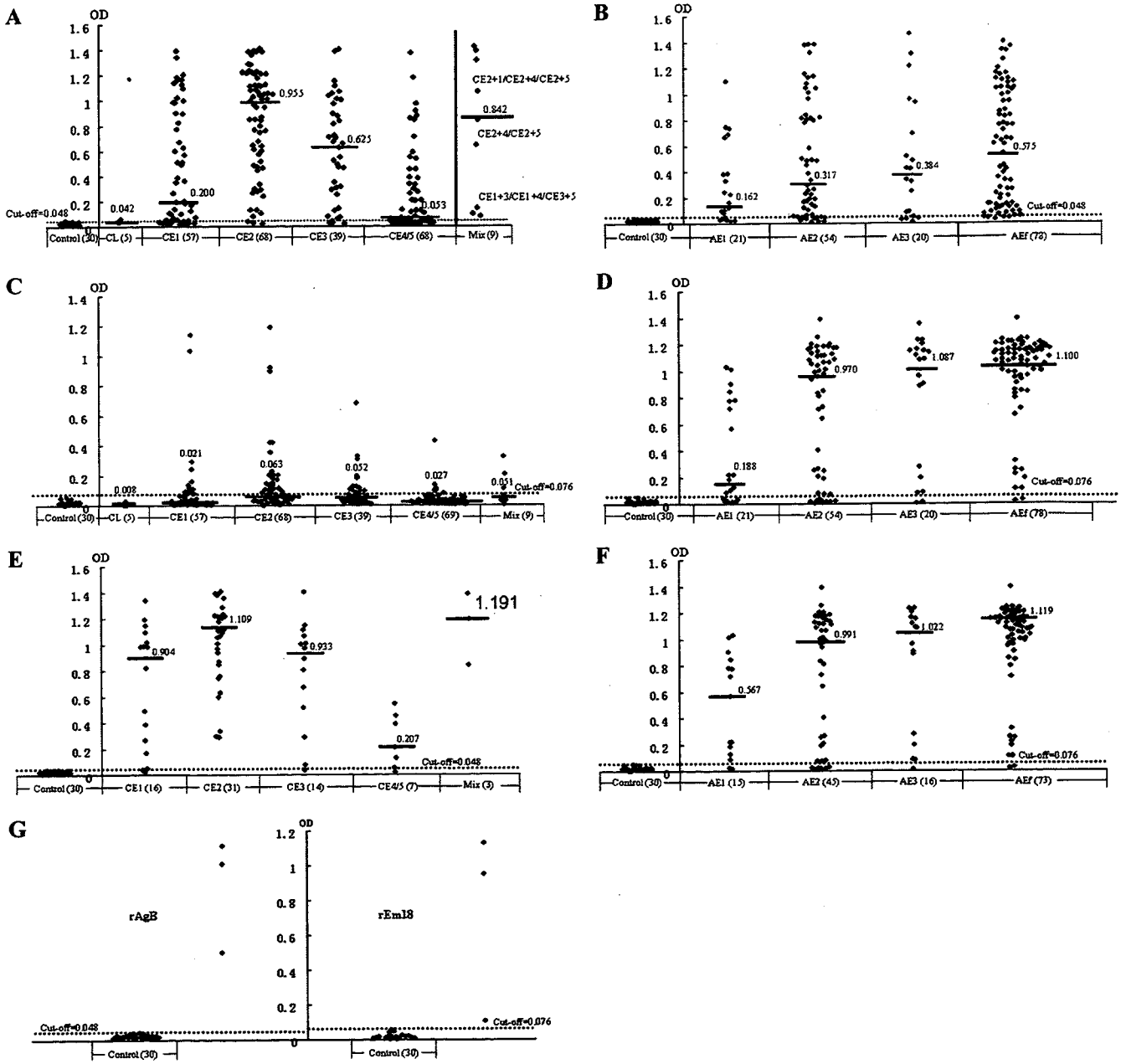

FIG. 1. Results of rAgB and rEm18 ELISAs for CE and AE patients with echinococcal lesions at different stages. (A) rAgB ELISA in 246 CE cases; (B) rAgB ELISA in 173 AE cases; (C) rEm18 ELISA in 246 CE cases; (D) rEm18 ELISA in 173 AE cases; (E) rAgB ELISA in 71 CE cases with a positive response to rEm18; (F) rEm18 ELISA in 149 AE cases with a positive response to rAgB; (G) rAgB and rEM18 ELISAs for 3 cases with dual infections of both CE and AE. The dashed lines indicate the cutoff values, and black bars refer to OD medians. Controls were healthy persons. The numbers in parentheses indicate numbers of tested cases or persons.

0.489 for the positive cases. Serum levels and concentrations of specific antibody were shown to be elevated in patients with late-stage disease (AE2, AE3, or AEf) (Fig. 1B). That is, 93.6% (73/78) of AEf patients exhibited a positive antibody response, while positive responses were observed in 83.3% (45/54) for AE2, 80.0% (16/20) for AE3, and 71.4% (15/21) for AE1 patients (Table 2). Further analysis revealed that the differences in the positive rates was significant ($\chi^2 = 8.41$; $P = 0.0382$).

TABLE 2. Results of ELISAs with rAgB and rEm18 as antigens in 173 AE patients

Cyst type	No. of patients examined	No. (%) of patients with positive response to:	
		rAgB	rEm18
AE1	21	15 (71.4)	14 (66.7)
AE2	54	45 (83.3)	43 (79.6)
AE3	20	16 (80.0)	18 (90.0)
AEf	78	71 (91.0)	76 (97.4)
Total	173	147 (85.0)	151 (87.3)

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(ii) rEm18 ELISA. Of the same 173 AE cases, 87.3% (151) exhibited a specific antibody response to rEm18, with an OD median of 1.068 for the positive cases. Antibody levels and concentrations were observed to be greatly elevated with advanced disease (Fig. 1D). That is, 14 (66.7%) of 21 patients with AE1-type lesions showed positive reactions, while 79.6% (43/54) of AE2 and 90.0% (18/20) of AE3 cases exhibited specific antibody, and the positive rate achieved 97.4% (76/78) in patients with AEf-type lesions (Table 2). The positive rate proved to be significantly different ($\chi^2 = 18.27$; $P < 0.0005$). In addition, the difference of OD medians between those patients at different stages (AE1, AE2, AE3, or AEf) was also significant ($\chi^2 = 32.265$; $P = 0.000$).

Of the 149 AE patients with a positive response to rAgB, 136 (91.3%) exhibited specific antibody to rEm18 (Fig. 1F). In other words, AE patients with a positive response to rEm18 were more likely to react with rAgB (136/151; 90.1%) than AE patients with a negative response to rEm18 (13/22; 59.1%) ($\chi^2 = 15.33$; $P < 0.0001$).

As expected, a much greater proportion of AE patients (87.3%) exhibited rEm18-specific antibodies than CE patients (28.9%; $\chi^2 = 138.83$; $P = 0.000$). Similarly, rEm18 ELISA OD values of AE patients (OD median, 1.029) were significantly higher than those of CE patients (OD median, 0.036; $P = 0.000$). In contrast, antibody activities with rAgB in CE patients (77.6%) or AE patients (86.1%) were different ($\chi^2 = 4.77$; $P = 0.0290$), but the difference of OD medians was not significant ($P = 0.473$).

Cases with dual infection. All three cases with dual CE/AE infection showed positive responses to both rAgB and rEm18 (Fig. 1G).

DISCUSSION

rAgB and rEm18 have recently been produced and have proved to be highly useful for serodiagnosis of human echinococcosis, with a high sensitivity and 100% specificity (26, 30, 37). Our current study focused on testing the sensitivity, and the results indicated that rAgB detected *Echinococcus* genus-specific antibodies, because both CE and AE patients were seropositive to a similar level, whereas rEm18 antigen exhibited higher *E. multilocularis* species specificity, with 87.3% of AE cases classified as seropositive whereas by contrast only weak reactions were observed in 28.9% of CE patients. In addition, specific IgG antibody levels and concentrations measured against rAgB and rEm18 proved to be strongly correlated with disease stage in CE and AE cases, respectively.

Both human CE and AE are highly endemic in northwest Sichuan Province, China (22, 23), where a large number of echinococcosis cases at different stages were detected in the field through mass screening programs by portable ultrasound scan, which permitted us to analyze the correlation of specific antibody response and disease stage. Considering the natural history of cystic echinococcosis, cysts are classified into six types; CL (refers to cysts of a parasitic origin), CE1, CE2, CE3, CE4, and CE5, indicating the different pathological/growth activities of the parasite in human hosts (36). Diagnosis of CE is currently primarily based on the imaging features of the cysts, but specific serology is also important as a complementary diagnostic tool. As one of the most important immunogenic antigens, *E. granulosus* native AgB detects about 80% to 90% of CE cases (15, 19, 24, 25), while rAgB has shown a similar diagnostic value, with positive reactions in about 70% to 90% of CE cases (26, 28, 33). Our current study revealed that rAgB had a similar positive rate (77.6%) in CE patients, and rAgB-specific antibody levels and concentrations in CE patients were strongly associated with the cyst type, i.e., when the parasite was at a very early (CL) or inactive (CE4 or CE5) stage, the specific IgG antibodies were present at a significantly lower concentration in a small proportion of patients, with a seropositive rate of 54.8% and an OD median of 0.050, compared to 87.3% seropositive and an OD median of 0.648 when the parasite was in an active (CE1 or CE2) or transitional (CE3) stage of development. Similar observations were made previously for ultrasound-confirmed CE cases detected in community studies (2, 3) and for hospitalized patients (28). Interestingly, patients with CE1 cysts in our study showed a markedly lower seropositivity (73.7%) and lower OD (median, 0.2) with rAgB than patients with CE2 cysts (95.6% seropositive and 0.995 OD median); this was probably caused by different structural features of the cysts, which can lead to the release of fewer antigens, including antigen B in the blood circulation, in CE1 cases than in CE2 cases. However, a similar seropositivity with native AgB in CE1 and CE2 cases was reported by Ortona et al. (28); this discrepancy might arise from differences in the time of serum sampling (before or after surgical or chemotherapeutic intervention), but it may be because CE2 and CE3 were revised in the original Gharbi classification (7, 35) before the WHO recommendation to change these criteria were published (36). In our study, 86.1% of AE sera were also recognized by rAgB, which was exceptionally higher than that where AE is exclusively endemic (approximately 40.0%), as reported previously (15, 19, 26). One possibility is that these AE cases might be coinfected with CE in other organs, such as the lung. However, a more likely probability is that rAgB applied in our study refers to rAg8/1 from *E. granulosus* protoscolices (rEgAgB8/1), which is 92.6% homologous at the amino acid level to AgB8/1 from *E. multilocularis* metacestodes (EmAgB8/1) (26). These two antigens have been shown to have very similar immunoreactive regions, which are thought to stimulate human hosts to produce similar IgG antibodies in CE and AE patients, respectively (26). Therefore, rAgB, although from *E. granulosus*, can bind IgG antibodies in both CE and AE sera to a similar level.

Several *E. multilocularis* antigens, such as EM10 (5), II/3 (34), II/3-10 (27), and EM4 (10), have proved to have potential for use in differential serodiagnosis of AE from CE. Em18 from *E. multilocularis* protoscoleces was confirmed to be a fragment of EM10 (30) and demonstrated its usefulness for highly sensitive and specific diagnosis of AE (12, 13, 14, 17, 18). In the current study, 87.3% of AE patients exhibited a specific antibody response to rEm18, which was identical to results of a previous report (30) but was lower than the results from other studies in which rEm18 detected almost 100% of AE cases (16, 37). This discrepancy is probably caused by the differences of the disease stages, as our study indicated the rEm18-specific antibody levels and concentrations in AE patients were strongly correlated with the stage of alveolar lesions. When the disease became aggravated, with the lesion changing from AE1 to AE2, AE3, or AEf, antibody activities

against rEm18 were significantly elevated, from 66.7% to 97.4%, and concurrently serum concentrations of specific antibody also evidently increased with the OD value, changing from 0.188 to 1.100. This observation indicates that Em18 can be highly useful for assessing the parasite activities in human AE patients following interventional measures. Similar results have recently been published in which rEm18 serology was shown to be reliable for monitoring the progression of AE (11, 31). A much greater proportion (28.9%) of CE cases were observed to exhibit specific antibody against rEm18 than those (3% to 13%) described in previous studies (16, 18, 30, 37), in which positive patients were found to have complicated or multiple cysts. In the current study, CE patients with all types of cysts except for CL were observed to respond to rEm18, of which cases with CE2 (45.6%) were most likely to have specific antibody compared to the other groups (ranging from 10.3% to 35.9%). Nevertheless, all the seropositive reactions in CE patients were much weaker than in AE patients (OD medians, 0.135 versus 1.068). Similar results were obtained with antigens EM10 or II/3, described in previous studies (4, 6), in which AE patients were found to have raised specific antibody to EM10 more frequently than CE patients, despite there being a protein with a high level of homology to EM10 expressed by *E. granulosus* metacestodes. The significant differences in activities with rEm18 or EM10 in AE and CE patients may be partially attributable to different pathological features of metacestodes of *E. multilocularis* and *E. granulosus*. The *E. granulosus* metacestode grows in a cyst with a double wall by endogenous budding. Conversely, the *E. multilocularis* metacestode grows via exogenous budding, and it therefore has intimate contact with host tissues (32).

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Widespread co-endemicity of human cystic and alveolar echinococcosis on the eastern Tibetan Plateau, northwest Sichuan/southeast Qinghai, China

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ABSTRACT

Cystic echinococcosis (CE) or hydatid disease is known to be cosmopolitan in its global distribution, while alveolar echinococcosis (AE) is a much rarer though more pathogenic hepatic parasitic disease restricted to the northern hemisphere. Both forms of human echinococcosis are known to occur on the Tibetan Plateau, but the epidemiological characteristics remain poorly understood. In our current study, abdominal ultrasound screening programs for echinococcosis were conducted in 31 Tibetan townships in Ganze and Aba Tibetan Autonomous Prefectures of northwest Sichuan Province during 2001–2008. Hospital records (1992–2006) in a major regional treatment centre for echinococcosis in Sichuan Province were also reviewed. Of 10,186 local residents examined by portable ultrasound scan, 645 (6.3%) were diagnosed with echinococcosis: a prevalence of 3.2% for CE, 3.1% for AE and 0.04% for dual infection (both CE and AE). Human cystic and alveolar echinococcosis in pastoral areas was highly co-endemic, in comparison to much lower prevalences in semi-pastoral or farming regions. The high ultrasound prevalence in these co-endemic areas in northwest Sichuan Province was also reflected in the hospital study, and hospital records furthermore indicated another possible highly co-endemic focus in Guoluo Prefecture of Qinghai Province, located at the border of northwest Sichuan. These chronic cestode zoonoses constitute an unparalleled major public health problem for pastoral Tibetan communities, and pose great difficulties for adequate treatment access and effective transmission control in such remote regions.

1. Introduction

Human echinococcosis refers to infection with the larval (metacestode) stage of zoonotic cestodes (tapeworms) belonging to the genus *Echinococcus*. Four main species were recognized until recently, namely, *Echinococcus granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli* (Rausch and Bernstein, 1997; Kumaratilake and Thompson, 1982). A new (fifth) species of *Echinococcus*, named *E. shiquicus*, has recently been described by our team in wildlife hosts from the eastern Tibetan Plateau, China (Xiao et al., 2005), however its infectivity to humans is unknown (Li et al., 2008). All the classic four recognized *Echinococcus* species of carnivores can infect humans (i.e. zoonotic) and may cause three clinical forms of echinococcosis, i.e. cystic echinococcosis (CE) caused by *E. granulosus*, alveolar echinococcosis (AE) caused by *E. multilocularis*, or polycystic echinococcosis due to *E. vogeli* or *E. oligarthrus*. The distribution of *E. granulosus* is cosmopolitan and is the predominant cause of human echinococcosis worldwide (McManus, 2002). Transmission of *E. oligarthrus* and *E. vogeli* is restricted to Central and South America where sporadic cases may occur, especially due to the latter species (D'Alessandro, 1997). *E. multilocularis* is also a relatively rare parasitic disease in humans and is restricted to the Northern Hemisphere, with primary transmission in wildlife (cycling between foxes and rodents). Human AE cases have however occurred more frequently in foci in Alaska, northern and central Europe, Central Asia, Siberia, China and Japan (Craig, 2003).

In China, human CE has been demonstrated to be widespread in at least 21 of its 31 provinces, but was more prevalent in the following northwest Provinces or Autonomous regions: Qinghai, Gansu, Sichuan, Ningxia, Xinjiang, Inner Mongolia, Tibet and Yunnan (Shi, 1997; Wen and Yang, 1997; Craig, 2004). From the 1990s active mass screening surveys using portable ultrasound began to reveal very high prevalence rates of human alveolar echinococcosis in several agricultural counties of Gansu and Ningxia provinces

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(Craig et al., 1992; Yang et al., 2006), and more surprisingly in pastoral Tibetan communities in western Sichuan Province (Li et al., 2005).

With the aim of understanding the epidemiology of human echinococcosis (both CE and AE) in Tibetan communities of the eastern Tibetan Plateau, mass screening programs for echinococcosis were conducted in 31 Tibetan townships located in western Sichuan Province. In addition, hospital records of echinococcosis patients post-operatively confirmed were reviewed from one of the main treatment centres in the region.

2. Materials and methods

2.1. Study sites

Parts of populations in a total of 31 townships, located in Ganze and Aba Tibetan Autonomous Prefectures of northwest Sichuan were screened by mass ultrasound. Participants in the study were selected on a voluntary basis. The involved townships included 24 pastoral townships within Ganze and located in the counties of Shiqu, Seda, Baiyu, Ganzi, Dege or Yajiang, where altitudes ranged from 3700 m to 4500 m, and the main occupation of local residents was raising yaks and sheep/goats as the primary source of income. In addition, three farming (primary agricultural) townships were selected from Yajiang and Danba Counties (Ganze Prefecture) and Maerkang County (Aba Prefecture), with altitudes varying from 2010 m to 2680 m. Four semi-pastoral townships were also included and were located in Rangtang County (Aba Prefecture), with altitudes ranging from 3451 m to 3600 m, where local people subsisted on both agricultural and livestock grazing (Fig. 1). Screening programs for abdominal echinococcosis using portable ultrasound (GE, LOGIQ α100, Wuxi, China) were performed in Spring or early Winter during 2001–2008 in the selected sites. In cooperation with County level Centers for Disease Control (CDC) and local health administrators (cadres), information about the purpose of the screening program was spread to the villagers and townships. Volunteers were self-selected by informed consent and were assured free diagnosis and medical treatment with long-term albendazole drug therapy for echinococcosis if diagnosis was indicated. Recommendation was also provided for possible surgical intervention (cyst/lesion removal or drainage) if appropriate. Persons with other infections or medical conditions were examined and referred to local health clinics for further investigation or treatment.

2.2. Questionnaire

For each self-selected participant a questionnaire was completed using Tibetan registration auxillaries, designed to obtain information on demographics, animal ownership and potential risk factors for echinococcosis including the source of drinking water, dog ownership, the frequency of dog contact, ownership of fox skin products etc.

2.3. Criteria for diagnosis and classification of echinococcosis

Diagnosis and classification of cystic echinococcosis (CE) was made using portable ultrasound according to the criteria proposed by the World Health Organization Informal Working Group on Echinococcosis for CE (Pawlowski et al., 2001). On the basis of conformational features of cysts, CE lesions (primarily in the liver) were differentiated into six types including CL, CE1, CE2, CE3, CE4, and CE5. In this study, CL cysts of a parasitic origin were exclusively counted, and CL cysts of a non-parasitic origin were excluded by comprehensive analysis of other factors, particularly such as the patient age, and partially by further observation during follow-up. In general, the CL type refers to a cystic lesion without clear rim indicating the parasite is at an early stage of development if the cyst is of a parasitic origin, while the presence of CE1 or CE2 is suggestive of active stages of the disease, while CE3 suggests the parasite is at

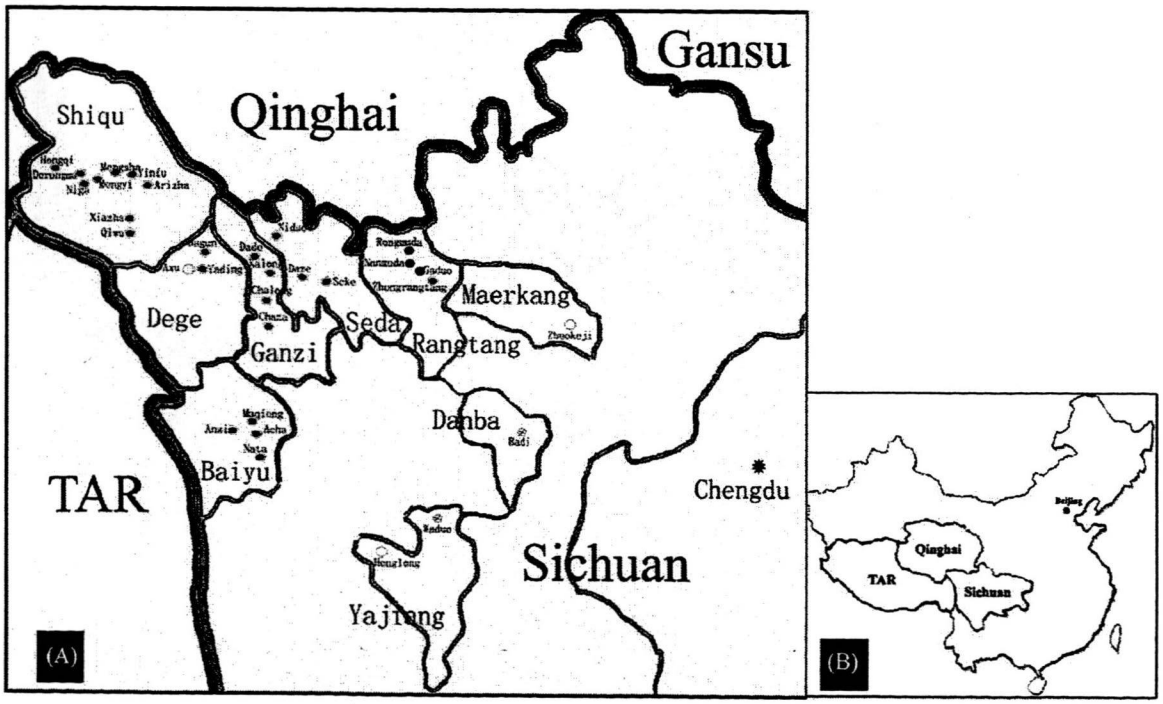

Fig. 1. Study sites of mass screening programs for abdominal echinococcosis by B-ultrasound in Sichuan Province, China. (A) Localities of study sites–(●) townships with detection of AE cases; (○) townships with detection of CE cases; (◉) townships with detection of both CE and AE cases; (⊗) townships without echinococcosis cases detected. (B) China map with the locality of Beijing (capital), Sichuan Province, Qinghai Province and Tibetan Autonomous Region (TAR).

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a transitional stage, and CE4 or CE5 implies an involution, necrotic or inactive parasite (Pawlowski et al., 2001).

Diagnosis of AE was dependent on detection of distinctive tumour-like lesions in the liver characterized by a non-homogeneous hyperechoic structure and with poorly defined verge, containing scattered calcifications, and/or a central necrotic cavity with a hyperechoic pseudoliquid structure (Pawlowski et al., 2001; Bresson-Hadni et al., 2006) and further classification was based on the criteria proposed previously (Li et al., 2004) and the PNM system (Kern et al., 2006). Briefly, on the basis of the features and the size of lesions, AE lesions were classified into three types and eight subtypes which indicated different stages of the disease. AE lesions ≤5 cm, normally without central necrosis detected, were confirmed as AE1 and differentiated further as, AE1s (single lesion) and AE1m (multiple lesions), which indicated an early stage of the disease. Alveolar lesions measured >5 cm ≤ 10 cm were classified as AE2 including three subtypes recorded as AE2s (single lesion), AE2m (multiple lesions) and AE2f (presence of central necrotic fluid, regardless of the number of lesions), suggestive of a developing parasite, while AE lesions measured >10 cm at diameter were confirmed as AE3 indicative of an advanced stage of the disease, which include three subtypes, i.e. AE3s (single lesion), AE3m (multiple lesions) and AE3f (presence of central necrotic fluid, regardless of the number of lesions).

2.4. Serology

Persons with confirmative or suspected CE/AE lesions or with other space-occupying lesions in the liver were asked to give a five ml venous blood sample for detection of *Echinococcus* antibodies using ELISA with recombinant AgB as antigen for CE or AE, and ELISA with recombinant Em18 antigen for AE, as described elsewhere (Sako et al., 2002; Xiao et al., 2003; Mamuti et al., 2004).

2.5. Hospital study

All patients who were treated surgically for echinococcosis in Aba Military Hospital (located in Maerkang, Aba Prefecture, Sichuan Province) (Fig. 5) during 1992–2006, and post-operatively confirmed by histopathology as echinococcosis, were included in this study ($n = 1312$). Further information about age, gender, domicile, ethnicity, and post-operative diagnosis, etc was collected. This hospital has 200 beds of which 95% are used to admit non-military patients.

Diagnosis of echinococcosis before operation was made by abdominal B-ultrasound examination and/or computed tomography (CT) (Ren et al., 2008). Post-operative confirmation was made by histopathology, as well as by PCR and DNA sequencing of isolates for some cases (Li et al., 2008). Briefly, morphological identification of CE infection was based on the observation of the structure of cystic lesions characterized by unilocular cysts with a thick laminated layer, presence of a germinal layer, and/or brood capsules with protoscoleces. For AE histopathology, the presence of large numbers of vesicles with different sizes and shapes with a thin laminated layer, and concurrence of distinct hyperplasia of fibro-connective tissue and cellular infiltration of eosinophils, lymphocytes and plasma cells, resulted in a diagnosis.

Table 1
Prevalence of human echinococcosis at township levels by ultrasound scanning in northwest Sichuan Province.

County	Township	No. examined	CE (*n*) %	AE (*n*) %	Dual infection	Total %
Shiqu	Niga	475	4.00(19)	4.00(19)	0	8.00
	Mengsha	356	9.55(34)	7.02(25)	1	16.85
	Yiniu	631	3.33(21)	9.35(59)	0	12.68
	Arizha	381	5.51(21)	8.14(31)	1	13.91
	Xiazha	584	7.02(41)	4.79(28)	0	11.82
	Qiwu	349	6.88(24)	1.72(6)	0	8.60
	Derongma	108	10.19(11)	4.63(5)	0	14.81
	Mengyi	100	14.00(14)	7.00(7)	0	21.00
	Hongqi	212	11.32(24)	5.66(12)	1	17.45
Ganzi	Kalong	549	0.91(5)	3.83(21)	0	4.74
	Chalong	614	2.61(16)	6.35(39)	0	8.96
	Chaza	116	2.59(3)	2.59(3)	0	5.17
	Dade	123	1.63(2)	3.25(4)	0	4.88
Seda	Daze	310	2.58(8)	3.23(10)	1	6.13
	Niduo	229	3.93(9)	1.75(4)	0	5.68
	Seke	492	0.61(3)	1.42(7)	0	2.03
Baiyu	Maqiong	302	3.97(12)	1.66(5)	0	5.63
	Nata	271	1.48(4)	2.58(7)	0	4.06
	Acha	258	3.10(8)	0.78(2)	0	3.88
	Anzi	119	1.68(2)	2.52(3)	0	4.20
Rangtang	Gaduo	274	0(0)	1.82(5)	0	1.82
	Nanmuda	145	0(0)	1.38(2)	0	1.38
	Rongmuda	162	0(0)	0.62(1)	0	0.62
	Zhongrangtang	94	1.06(1)	2.13(2)	0	3.19
Dege	Dagun	269	3.35(9)	0.37(1)	0	3.72
	Yading	198	7.07(14)	1.52(3)	0	8.59
	Axu	117	5.13(6)	0(0)	0	5.13
Yajiang	Honglong	610	2.30(14)	0(0)	0	2.30
	Waduo	584	0(0)	0(0)	0	0.00
Maerkang	Zhuokeji	571	0.88(5)	0(0)	0	0.88
Danba	Badi	583	0(0)	0(0)	0	0
Total		10186	3.24(330)	3.05(311)	4	6.33

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2.6. Statistical analysis

Data were analyzed using Epi InfoTM (version 3.5; Centers for Disease Control and Prevention, Atlanta, GA), statistical significance was set at $p < 0.01$.

3. Results

3.1. Mass screening using ultrasound

A total of 10,186 participants originating from Tibetan communities of 31 townships in nine counties in Sichuan Province were registered by questionnaire and examined by abdominal ultrasound. Population sample (age ranged from 1 to 92 years; median 32.8 years) comprised 50.4% (5133) females and 49.6% (5053) males. Persons of Tibetan ethnicity comprised 96.1% of the sampled population. The other participants listed their ethnicity as Han (3.9%). Questionnaire data revealed 46.9% (4781) were herdsmen who raised livestock including yaks, sheep and/or goats as the primary source of their income. Other listed occupations included student (19.1%), farmer (12.2%), part-time herdsman (4.5%), public servant (7.4%), preschooler (3.6%), and other (6.3%).

3.1.1. Prevalence of human echinococcosis

Of 10,186 volunteers examined by abdominal ultrasound scanning, 311 (3.1%) were confirmed to have AE infection, 330 (3.2%) to have CE, and 4 (0.04%) individuals to have dual infection with both CE and AE (Table 1; Figs. 2 and 3).

Of 315 persons with a confirmative image of AE lesions (including four persons with dual infection), 74 (23.5%) had hepatic lesions of AE1 type (67 AE1s and 7 AE1m), 142 (45.1%) had AE2 lesions in the liver (75 AE2s, 20 AE2m and 47 AE2f), while hepatic lesions characterized by AE3 were detected in 99 (31.4%) individuals (34 AE3s, 3 AE3m and 62 AE3f) (Table 2). In addition, 136 single AE lesions were located in the right hepatic lobe, and 36 were in the left hepatic lobe. Involvement of both lobes was observed in 79 AE cases. More than one alveolar lesion was detected in the liver in the remaining 64 cases.

Of 334 persons who presented with cystic images indicative of CE cysts (including 4 cases with dual infection), nine (2.7%) were detected to have CL type lesions, 103 (30.8%) were classified to have CE1 lesions, 98 (29.3%) had CE2 cysts, 12 (3.6%) had CE3 lesions, 80 (24.0%) and 17 (5.1%) had CE4 or CE5 type cysts, respectively. Furthermore, multiple CE lesions with mixed type cysts were detected in an additional 15 cases (4.5%), i.e. 5 patients with CE1 and CE2 cysts, 3 with CE1 and CE4 cysts, 5 with CE2 and CE4 cysts, 1 with CE2 and CE5 cysts, and 1 with CE3 and CE5 type cysts (Table 2). In addition to the liver, one or more CE cystic lesions were identified in the peritoneal cavity in 24 patients, 7 cases in the spleen and 1 case in the kidney.

3.1.2. Echinococcosis prevalence at township level

Of 7773 volunteers originating from 24 pastoral townships that were examined by ultrasound, 324 (4.2%) were confirmed to have CE infection, 301 (3.9%) to have hepatic AE infection, and 4 (0.04%) persons presented with dual infection (both CE and AE). The overall prevalence of echinococcosis in pastoral townships ranged from 2.0% (10/492) in Seke Township, Seda County to 21.0% (21/100) in Mengyi Township, Shiqu County. The highest AE prevalence (9.4%, 59/631) was recorded in Yiniu Township of Shiqu County, and the highest CE prevalence (14.0%, 14/100) was recorded in Mengyi also in Shiqu County. No AE cases were detected in Honglong Township,

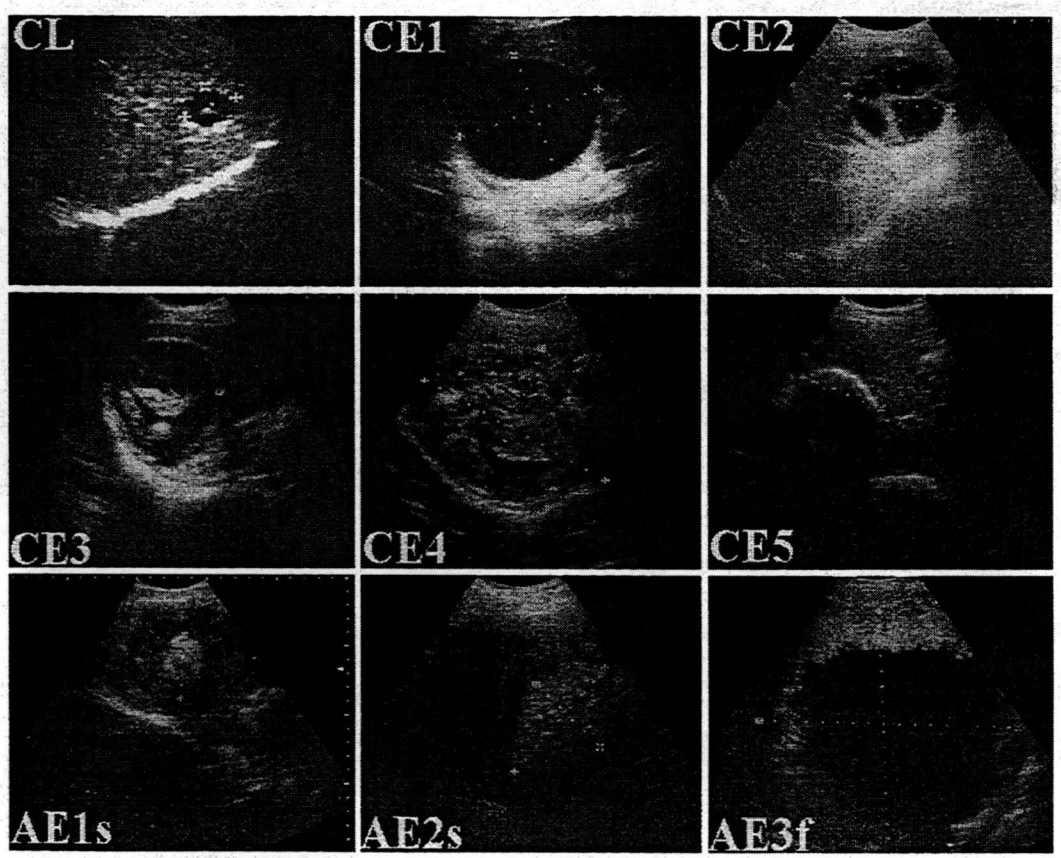

Fig. 2. Ultrasound images of different types of hepatic echinococcal lesions in patients detected in northwest Sichuan.

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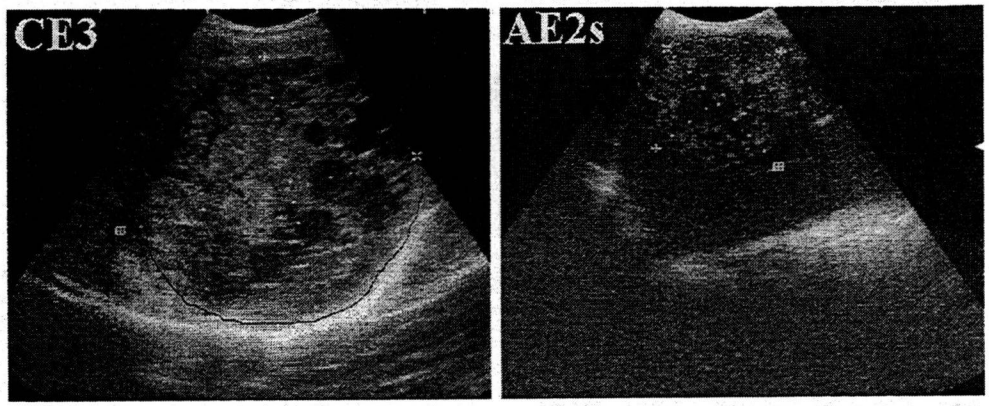

Fig. 3. Hepatic ultrasound images of the patient with dual infection (both CE and AE) in northwest Sichuan Province. CE and AE lesions were marked by a circle, respectively.

Yajiang County, however CE prevalence was 2.3% (Table 1). Therefore, both human cystic and alveolar echinococcosis was shown to be highly co-endemic in 23 of 24 pastoral townships studied in western Sichuan (Table 1).

In contrast, a much lower prevalence of human echinococcosis was recorded in farming or semi-farming townships, i.e. only 5 CE cases (3 CE4 and 2 CE5 advanced type cysts) were detected in 3 farming townships, with a prevalence of 0.3% (5/1738). Furthermore, only 0.1% (1/675) and 1.5% (10/675) of local people in 4 semi-farming townships had CE or AE infection, respectively (Table 1).

3.1.3. Prevalence by gender and age

Of 645 persons with an evidence of abdominal echinococcosis, 394 (CE = 212, AE = 179, dual infection = 3) were female, and 251 (CE = 118, AE = 132, dual infection = 1) were male. In other words, the prevalence of echinococcosis (CE or AE) in females was 7.7% (394/5133) (4.1% for CE, 3.5% for AE and 0.1% for dual infection), and 5.0% (251/5053) for male (2.3% for CE, 2.6% for AE and 0.02% for dual infection). Statistical analysis revealed that the prevalence of echinococcosis in females (7.7%) was significantly higher than that for males (5.0%) ($\chi^2 = 31.49$, $p < 0.01$).

Among the 645 persons with abdominal detectable *Echinococcus* lesions, the average age was 40.9 years. The youngest patient with CE was 3 years and the oldest was 81 years, and the average age of CE cases detected by ultrasound was 38.6 years ($n = 330$). However, age specific prevalence of CE cyst type varied. The average age of subgroup population with CE cysts at an early stage (CL) lesions was 11.3 years ($n = 9$), while average age group with CE cystic lesions at active stages (CE1, CE2), at transitional stages (CE3) or inactive stages (CE4, CE5), was 38.2 years ($n = 201$), 36.3 years ($n = 12$) and 43.5 years ($n = 97$), respectively. The average age of persons diagnosed in the communities with hepatic AE was 43.4 years (age range 6–81 years). The subgroup of cases with early AE1 lesions had an average age of 39.7 years ($n = 74$), while the mean age of the population with AE2 lesions was 44.7 years ($n = 142$), and 44.0 years for the subgroup with advanced AE3 lesions ($n = 99$). In addition, the average age for 4 persons with dual infection was 39.8 years (ranging 11–66 years).

For human AE prevalence showed an increase with age, which peaked at the >60 years group (6.5%). Further analysis revealed a significant difference of AE prevalence between age groups ($\chi^2 = 108.91$, $p < 0.01$, 6 degrees of freedom). For abdominal CE prevalence peaked at 5.0% in the >40 to ≤50 years age group, and then decreased. The four persons with dual CE and AE infection were detected across the age cohort (Fig. 4).

3.1.4. Prevalence of echinococcosis by occupation

Herdsman had the highest risk for abdominal echinococcosis infection, with a total prevalence of 10.6% (505/4781) (5.5% for CE and 5.0% for AE). Farmers had the lowest prevalence of 0.4% (5/1245) (0.4% for CE). For other occupations, the prevalence was recorded as 2.2% (10/453) for part-time herdsman (0.2% for CE and 2.0% for AE), 1.7% (34/1944) for students (0.9% for CE, 0.8% for AE and 0.05% for dual infection), 1.9% (7/371) for preschooler (1.6% for CE and 0.3% for AE), 3.9% (29/749) for public servants (2.0% for CE and 1.9% for AE), and 8.6% (55/643) for others (3.6% for CE, 4.8% for AE and 0.2% for dual infection). The prevalence for both CE and AE combined proved to be of statistical significance between occupations ($\chi^2 = 325.28$, $p < 0.01$, 6 degrees of freedom).

3.2. Serology

A total of 191 (77.6%) CE sera and 147 (85.0%) AE sera showed positive response to *E. granulosus* recombinant antigen B (rAgB), while 28.9% (71/246) CE sera and 87.3% (151/173) AE sera were recognized by *E. multilocularis* recombinant Em18 (rEm18). Moreover, significant differences of seropositivities with ELISA-rAgB

Table 2
Ultrasonic classification of echinococcal lesions in 649 cases (including 4 patients with dual infection) in northwest Sichuan Province.

Subtype of AE	No. cases	Proportion %	Subtype of CE	No. cases	Proportion %
AE1s	67	21.27	CL	9	2.69
AE1m	7	2.22	CE1	103	30.84
AE2s	75	23.81	CE2	98	29.34
AE2m	20	6.35	CE3	12	3.60
AE2f	47	14.92	CE4	80	23.95
AE3s	34	10.80	CE5	17	5.09
AE3m	3	0.95	Mix	15	4.49
AE3f	62	19.68			
Total	315	100.00		334	100.00

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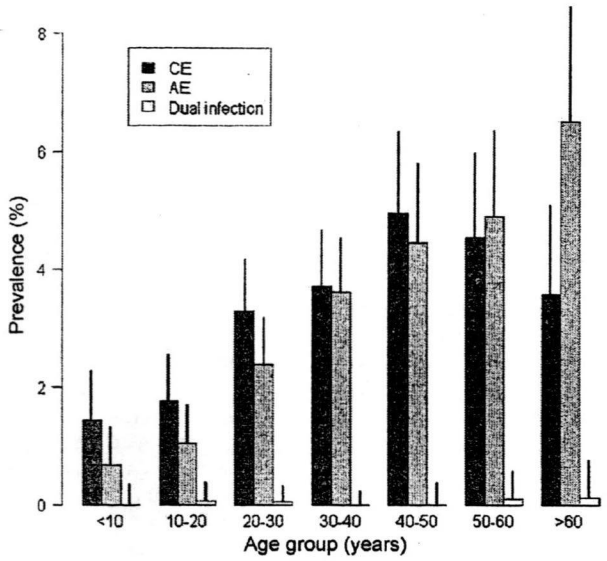

Fig. 4. Prevalence of echinococcosis by age groups in northwest Sichuan Province.

or ELISA-rEm18 were observed in CE/AE cases with echinococcal lesions at different stages. Detailed description of the serology profiles will be reported elsewhere (Li et al., unpublished data).

3.3. Aba hospital study

During 1992–2006, records were identified for 1312 patients (male = 719, female = 593) with post-operatively confirmed echinococcosis in Aba Military Hospital. The majority 79.6% (n = 1044) were confirmed as CE, and 268 as AE infection. Persons of Tibetan ethnicity comprised 97.4% (1278/1312) of these patients, and all the others were Han Chinese. The mean age of 1292 patients (information about age was not available for the other 20 patients) was 42.8 years, ranging from 4 to 81 years. The youngest CE case was 4 years old and the oldest 81 years, with a mean age of 42.9 years (n = 1027). While the average age of AE cases was 42.5 years (n = 265), with an age range of 8–79 years. The main treatment used for CE was endocystectomy, and for AE cases resection of total/partial alveolar lesion.

Of 1044 patients treated with CE infection, 84.8% (885) had cystic lesions located in the liver, 11.7% (122) in the abdominal cavity or pelvic cavity, and 1.6% (17) in both abdominal cavity and pelvic cavity as well as the liver. Involvement of other organs or tissues was also recorded: spleen CE (1 case), subcutaneous CE (8 cases), lung (4 cases), brain (3 cases), vertebra (2 cases), bone (1 case), and eye (1 case).

Of 268 AE cases treated, alveolar lesions were detected only in the liver in 93.7% (251) of patients. In the remaining 17 AE patients, other organs were also involved, i.e. the brain (5 cases), lung (5 cases), brain and lung (1 case), vertebra (5 cases) and bladder (1 case).

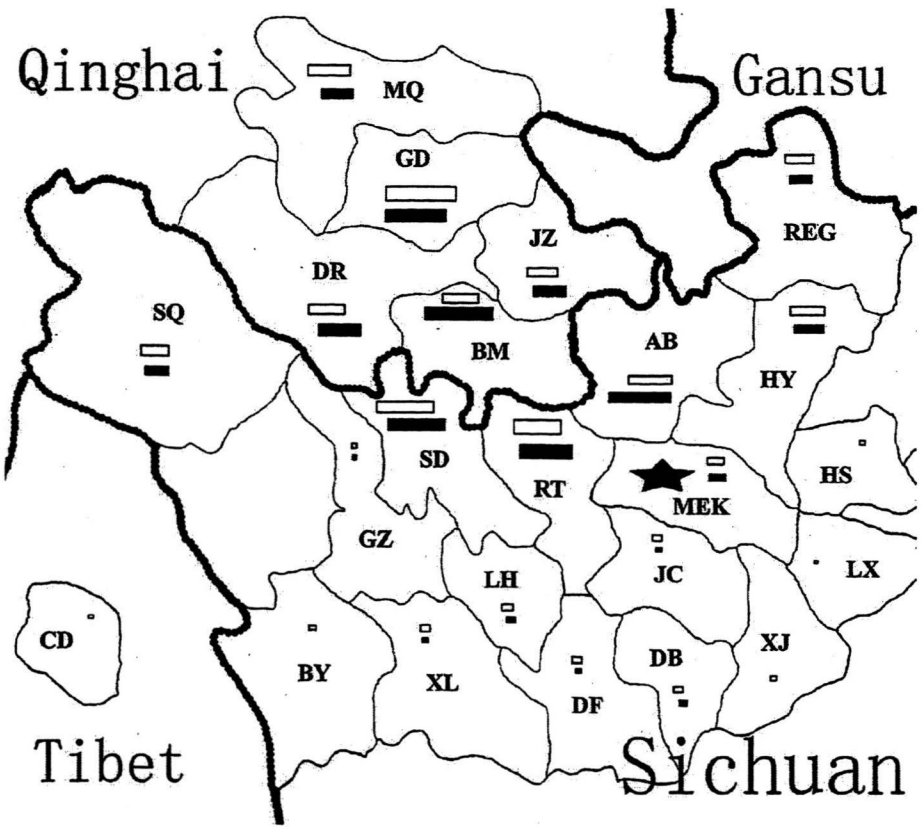

Fig. 5. Geographic distribution of 1312 human cases of echinococcosis post-operatively confirmed during 1992–2006 in Aba Army Hospital; Solid and open bars indicate AE and CE cases, respectively. The length of bars shows approximate proportion of each disease in the county. Symbol (*) shows the locality of Aba Army Hospital. Full name of each county is shown as follows: SQ = Shiqu, GZ = Ganzi, SD = Seda, BY = Baiyu, XL = Xinlong, LH = Luhuo, DF = Daofu, DB = Danba, RT = Rangtang, AB = Aba, REG = Ruoergai, MEK = Maerkang, HY = Hongyuan, HS = Heishui, LX = Lixian, XJ = Xiaojin, JC = Jinchuan, BM = Banma, JZ = Jiuzhi, GD = Gande, MQ = Maqin, DR = Dari, CD = Changdu.

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The majority (99.9%) of the 1312 echinococcosis patients treated at Aba Military Hospital lived in Sichuan or Qinghai provinces, and only one CE patient came from the Tibetan Autonomous Region (Changdu County) (Fig. 2). For 1044 CE cases, 56.5% (590) originated from 17 counties of Sichuan Province. The remaining 453 (43.4%) CE cases came from 5 counties of Guoluo Prefecture of Qinghai Province (Fig. 5). Of 268 AE cases treated at Aba Military Hospital, 150 (56.0%) resided in 13 counties of Sichuan Province, while 118 (44.0%) AE cases lived in the same 5 counties of Guoluo Prefecture, Qinghai Province, i.e. Banma, Dari, Gande, Jiuzhi, and Maqin counties (Fig. 5).

4. Discussion

Human cystic echinococcosis (CE) caused by *E. granulosus*, results in more lost DALYs (disability adjusted life years) globally than that due to onchocerciasis or Chagas disease (Budke et al., 2006), with the greatest echinococcosis burden in Central Asia and China (Craig et al., 2007). The Chinese Ministry of Health carried out a national survey in 2002 for 8 important parasitic diseases, and found the prevalence of human CE was highest (2.5%) in Tibetan communities (Craig et al., 2008). In western China human alveolar echinococcosis, caused by *E. multilocularis*, is also endemic albeit with a more focal distribution than CE and generally considered mainly to be a zoonotic disease in poor Han or Hui upland agricultural communities (Craig et al., 1992; Yang et al., 2006). Human CE and AE cause chronic cystic or vesicular lesions respectively, which may eventually be highly pathogenic and very difficult to treat (WHO, 1996; McManus et al., 2003).

We now demonstrate the occurrence of a major co-endemic focus of human CE/AE in Tibetan pastoral communities present in northwest Sichuan and southeast Qinghai at the eastern edge of the Tibetan Plateau. The average total ultrasound prevalence of human abdominal echinococcosis in 10,186 persons screened in two Tibetan Autonomous Prefectures of northwest Sichuan was 8.1% (4.2% for CE, 3.9% for AE, and 0.05% for dual CE/AE infection). The highest co-endemicity (10.6% total prevalence) at community level occurred in pastoralists (herdsmen occupation), in which approximately half the cases detected were due to the most pathogenic form AE. Human echinococcosis cases were also detected after mass screening in agricultural/semi-farming Tibetan communities but with significantly lower ultrasound prevalence (<0.5% for either disease). This high co-endemicity for CE and AE at pastoral community level in northwest Sichuan, was also verified by examination of hospital records in one important treatment centre in Aba Tibetan Autonomous Prefecture, and also identified CE and AE cases originating from another highly co-endemic pastoral region, i.e. Guoluo Tibetan Autonomous Prefecture in Qinghai Province (Han et al., 2006; Yu et al., 2008), which borders northwest Sichuan.

A total of 645 cases of human cystic ($n = 330$) and alveolar ($n = 311$) echinococcosis, as well as 4 mixed CE/AE cases, were identified by mass ultrasound screening in 8 counties of west Sichuan Province, i.e. counties of Shiqu, Seda, Ganze, Baiyu, Dege, Yajiang, Rangtang and Maerkang. Patient records from Aba Military Hospital in Maerkang (Sichuan) between 1992 and 2006, for 1312 confirmed echinococcosis cases ($n = 1044$ CE, $n = 268$ AE), identified patient domicile in an additional 5 counties in neighbouring Qinghai Province, i.e. counties of Banma, Dari, Jiuzhi, Gande and Maqin. More detailed analysis of these hospital cases has been reported elsewhere (Ren et al., 2008).

Western China has long been known to be endemic for human cystic echinococcosis (CE) (Craig et al., 1991), however the distribution of human alveolar echinococcosis (AE) the most pathogenic form, appears more focal with apparent hotspots in Gansu, Ningxia, Xinjiang, Qinghai and Sichuan provinces/regions, some of which are also co-endemic for CE (Craig et al., 1992; Yang et al., 2006; Li et al., 2005; Han et al., 2006; Yu et al., 2008; Schantz et al., 2003; Zhou et al., 2000). Human CE and AE are co-endemic in only a few other world regions, notably eastern Turkey, Central Asia and Siberia (Craig, 2003). The current study now identifies a geographic area on the eastern edge of the Tibetan Plateau of approximately 313,200 km^2 involving at least 11 pastoral counties over 2 adjacent provinces, with the greatest levels of co-endemicity of human CE and AE so far described worldwide.

In Tibetan pastoral communities, domestic dogs are kept in large numbers to guard property and livestock, and are usually tied during daytime and released at night. As Tibetan Buddhism forbids killing animals including dogs, with exception of food provision, large populations of stray dogs exist especially around temples. During the livestock slaughtering season (October to December), dogs (both owned and stray) are frequently fed with raw offal (including livers and lungs of yaks, sheep or goats) by herdsman. In addition, dogs may also prey on small mammals in around townships and adjacent pastures. A necropsy study of stray dogs in Ganze Prefecture revealed a 29.5% prevalence for *E. granulosus* and 11.5% for *E. multilocularis* (Qiu et al., 1989). A recent diagnostic purgation study in Shiqu County identified 8% of owned dogs infected with *E. granulosus* and 12% with *E. multilocularis* infection (Budke et al., 2005). The Tibetan fox (*Vulpes ferrilata*) appears to be the main sylvatic definitive host of both *E. multilocularis* and *E. shiquicus* in these pastoral areas (Qiu et al., 1995; Xiao et al., 2005), and ownership of fox skins was shown to be a risk factor for human AE (Wang et al., 2006). To date however, there is no evidence that human echinococcosis can be caused by *E. shiquicus* (Li et al., 2008). Yaks as well as sheep and goats appear to be key intermediate hosts for transmission of *E. granulosus* on the Tibetan Plateau (He and Liu, 2000). In addition, the high altitude grassland is abundant in small mammal communities, and up to 5 species (in the genera *Microtus*, *Cricetulus* and *Ochotona*) have so far been identified as possible key reservoir intermediate hosts of *E. multilocularis* (Giraudoux et al., 2006). The involvement of dogs as well as foxes in transmission of *E. multilocularis*, the diversity of small mammal potential hosts, landscape/habitat ecology of small mammals, together with poor hygiene and other risk behaviors (Li et al., 2005; Wang et al., 2006), seem to be major factors contributing to the high prevalence of both human cystic and alveolar echinococcosis in pastoral areas of the eastern Tibetan Plateau.

Landscape ecology suitable for transmission of *E. multilocularis* can vary over short distances (10 km) (Giraudoux et al., 2003). An interesting observation in the current study was the absence of AE cases detected in one pastoral township (Honglong in Yajiang County), where altitude (4168 m), average number of dogs owned (1.2) and livestock ownership, was similar to other pastoral townships with high human AE prevalence (e.g. Yiniu Township in Shiqu County at 4200 m and average 2.9 dogs owned per household). Subtle differences in landscape ecology could affect suitable habitat for potential small mammal hosts and their population dynamics which enable transmission of *E. multilocularis* (Giraudoux et al., 2003).

Although a human CE prevalence of 0.3% (mean age = 61.6 years) was found after ultrasound screening in farming areas, all cysts were confirmed by ultrasound to be involutive or inactive, i.e. CE4 or CE5 types, which may indicate less recent transmission of *E. granulosus* in those non-pastoral areas. Furthermore, no human AE cases were detected by mass screening in such farming areas. By contrast, upland farming/agricultural communities have been identified as important AE foci in some other parts of China (Craig et al., 1992) as well as in Europe (Giraudoux et al., 2003). In mixed farm-

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ing/pastoral Tibetan areas both CE and AE were detected during screening, but the prevalence was lower (0.2% for CE and 1.5% for AE), in comparison with the higher altitude pastoral regions. Traditionally, females in Tibetan communities are usually responsible for home chores, such as feeding dogs and collecting yak dung for fuel. Women may therefore have more opportunities to be exposed to environments contaminated by *Echinococcus* spp eggs, resulting in the significantly higher prevalence we observed in females. Older age groups of either sex, are at greater risk probably because they have more opportunities for exposure over time.

Portable ultrasound has been applied for community screening for abdominal echinococcosis in China since the early 1990s (Craig et al., 1992; Yang et al., 2006). Cases identified in remote rural areas have benefited from early diagnosis (Bartholomot et al., 2002; Li et al., 2005), which improves chances for better treatment and prognosis. Classification of ultrasound images of CE based on the criteria proposed by WHO provided information about the stage of this disease as well as choice of treatment procedures (WHO IWG, 2003). In the current study, patients with diagnosis of CE cysts that were classified on ultrasound image as CL (of a parasitic origin), CE1, CE2 or CE3, were considered to be active and growing and therefore recommended for either long-term oral albendazole therapy (6 months) with periodic follow-up, and/or for surgical treatment (endocystectomy or percutaneous approaches). Conversely, for patients with CE4 or CE5 hydatid cysts indicative of an involutive or inactive parasite, no positive therapy was considered necessary, rather a 'wait and watch' approach was adopted (Kern, 2003). For classification of alveolar echinococcosis cases the PNM system proposed by the WHO Informal Working Group for clinical settings (Kern et al., 2006) was applied. In addition, criteria for the classification of ultrasound images of alveolar echinococcosis was proposed by us for application in resource poor community settings (Li et al., 2004). In the current study, 31.4% of AE cases detected were categorized as AE3 (lesions measured >10 cm), indicative of an advanced stage with poor prognosis, because of impossibility of radical removal of alveolar lesions and poor efficacy of albendazole on late stage lesions (Pawlowski et al., 2001; Kern et al., 2006). AE cases that exhibited a lesion with a central necrotic cavity was detected in a total of 109 AE patients classified as AE2 or AE3, however, the presence of central necrosis was more frequent in cases with AE3 lesions (62.6%, 62/99) compared to those with AE2 type lesions (33.1%, 47/142) ($\chi^2 = 20.45$, $p < 0.01$). We also identified 4 cases that exhibited dual hepatic infection with both cystic and alveolar echinococcosis in the current study; an unusual and complicated clinical situation, and though rare, has been previously reported in western China (Wen et al., 1992; Yang et al., 2008).

In conclusion, a large co-endemic focus of human cystic and alveolar echinococcosis has been confirmed on the eastern Tibetan Plateau within Tibetan communities, primarily covering the border region of northwest Sichuan and southeast Qinghai, with an overall combined CE/AE prevalence of 6.3%. Prevalence was significantly higher in pastoral communities (8.1%) compared to that in semi-pastoral (1.6%) or farming communities (0.3%). Further active screening programmes and early surgical and/or medical interventions, particularly for alveolar echinococcosis, are imperative to reduce the public health impact of human echinococcosis in this remote resource-poor region of western China.

Finally, the initiation in 2006 of a national echinococcosis control programme (focused on dog dosing with praziquantel and active mass screening) which has so far covered 114 counties in western China, may succeed in interrupting transmission and reducing the public health impact of echinococcosis, but needs to be permanently implemented in these co-endemic areas of the eastern Tibetan Plateau.

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