E. granulosus s.s. is composed of three closely related genotypes, G1–G3. The common sheep strain (G1) is known to be highly infective for humans. It is reported from a wide range of intermediate host species, although fertile cysts are most common in sheep. The strain clusters firmly with Tasmanian sheep strain (G2) and buffalo strain (G3). All three variants occur sympatrically and do not show any obvious intermediate host specificity (Thompson 2008). The more distantly related genotype cluster G6–G10 (*E. canadensis*) includes strains found in wildlife in the northern Palearctic, but also the 'domestic' camel strain (G6) of the Middle East and northern Africa, and the pig strain (G7) of central-eastern and southern Europe. All are infective to humans, but reported cases are much rarer than those with G1.

Exposure of humans to CE is common in Turkey as the majority of people live in rural areas and is engaged in animal husbandry. Once a year, a sacrificial festival is held, during which a large number of sheep and cattle is slaughtered, with meat of infected intermediate host being commonly fed to stray dogs (Altintas 2003). Livestock is slaughtered also for daily meat requirements, and there is a high proportion of uncontrolled slaughter that facilitates the transmission of these parasites. The reported prevalence of CE in domestic animals in Turkey ranges from 3.5% to 58.6%, varying widely among locations, with a higher overall prevalence in sheep compared to goats, cattle and water buffaloes (Umur 2003; Altintas 2008a). Numerous human cases are regularly reported from medical centers in different parts of the country. According to Ministry of Health records, 52,124 patients had operations to treat or confirm CE in the period 1990–2005 which corresponds to 3,257 patients per year. The estimated incidence rate of CE in Turkey is 0.8–2.0/100.000 over the last years (Yolasigmaz et al. 2006; Altintas 2008b).

Despite its public health impact, relatively little information is available about the genetic variation of *Echinococcus* in Turkey. In a study of Obwaller et al. (2004), heterogeneity between the original PCR products and subclones of 16 *E. granulosus* isolates, including five Turkish isolates, was established. All isolates from Turkey corresponded to the G1–G3 cluster in the *cox1* gene, with two haplotypes detected within the studied sample. Later, Vural et al. (2008) also reported G1 and G3 in Turkey based on *cox1* sequences. The prominent occurrence of *E. granulosus* s.s. in the country was further confirmed by PCR-RFLP of a ribosomal ITS1 fragment and *cox1* sequencing (Utuk et al. 2008). The same species was reported from Turkish patients also in a methodological study of Schneider et al. (2008).

In neighboring Greece, G1 and G3 were found to be frequent and fertile in sheep, while fertile G7 was only recorded from goats (Varcasia et al. 2007). To the east of Turkey, isolates from Iran could be allocated to two groups corresponding with the G1 and G6 genotypes (Zhang et al. 1998; Fasihi Harandi et al. 2002; Ahmadi and Dalimi 2006). The common sheep strain (G1) was found to be the prevailing genotype, affecting sheep, cattle, goats, humans and occasionally camels. In these reports, the camel strain (G6) was recorded in most of the infected camels as well as in three of 33 human cases (Fasihi Harandi et al. 2002).

The aims of the study were to characterize Turkish isolates of the *E. granulosus* complex from humans and domestic animals using sequences of four mitochondrial genes, to allocate the isolates to the described strains, and to estimate the genetic variability within strains by characterizing their microvariants.

Materials and methods

Hydatid cysts of *E. granulosus* from sheep were collected from abattoirs in western Turkey (Izmir, Menemen-Izmir, Manisa, Denizli, Uşak) or were surgically excised or drained from CE patients resident in Atatürk Research and Training Hospital, Izmir, Turkey. Cyst contents were examined under light microscopy for the presence of protoscoleces. These were rinsed in physiological saline and stored in 90% ethanol until DNA isolation. Total genomic DNA was extracted from protoscoleces using DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Fragments of 4 genes of mitochondrial DNA were amplified using previously published oligonucleotide primers (Table 1). Two different portions of cytochrome *c* oxidase subunit 1 (*cox1*) were amplified at different laboratories. For a shorter fragment (366 bp), the cyclic PCR conditions were as follows: an initial denaturation step at 94°C for 30 s; 30 cycles at 94°C for 30 s, 52°C for 40 s, and 72°C for 45 s, followed by a final extension step at 72°C for 5 min. A longer *cox1* fragment (789 bp) was amplified under the following conditions: a host start 94°C for 30 s; 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The same PCR conditions were used for amplification of a portion of ATPase subunit6 (*atp6*) gene. A portion of the gene coding for NADH dehydrogenase subunit 1 (*nad1*) was amplified in following thermal conditions: a hot start at 94°C for 30 s; 35 cycles at 94°C for 30 s, 50°C for 45 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The cycling parameters for amplification of a small subunit (12 S) rRNA gene (*rrnS*) were: a hot start 94°C for 30 s; 30 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 5 min.

PCR products were visualized after electrophoresis in ethidium-bromide-stained 1.5% (*w/v*) agarose gels and

Table 1 Primer pairs used for PCR amplification of mitochondrial markers

Target	Sequences of primer pairs	Length of fragment (bp)	Reference
cox1 (longer fragment)	F: 5′-TTGAATTTGCCACGTTTGAATGC-3′ R: 5′-GAACCTAACGACATAACATAATGA-3′	789	Xiao et al. (2003)
cox1 (shorter fragment)	F: 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ R: 5′-TAAAGAAAGAACATAATGAAAATG-3′	366	Bowles et al. (1992)
atp6	F: 5′-GCATCAATTTGAAGAGTTGGGGATAAC-3′ R: 5′-CCAAATAATCTATCAACTACACAACAC-3′	513	Xiao et al. (2005)
nad1	F: 5′-AGATTCGTAAGGGGCCTAATA-3′ R: 5′-ACCACTAACTAATTCACTTTC-3′	471	Bowles and McManus (1993)
rrnS	F: 5′-TTAAGATATATGTGGTACAGGATTAGATACCC-3′ R: 5′-AACCGAGGGTGACGGGCGGTGTGTACC-3′	311	Dinkel et al. (1998)

purified using a Nucleospin Extract II kit (Macherey Nagel, Düren, Germany). Amplicons were directly sequenced using a dye terminator cycle sequencing kit (DYEnamic ET terminator; Amersham Biosciences, UK) and analyzed with an ABI PRISM 377 automated sequencer (Applied Biosystems, USA). Nucleotide sequences were aligned using Clustal W (Thompson et al. 1997) and compared to those stored in GenBank using BLAST program. Dendrograms were generated by MEGA version 3.1 software with neighbor-joining method using Kimura-2-parameter model (Kumar et al. 2004). Bootstrap analyses were conducted by using 1,000 replicates. To distinguish synonymous and non-synonymous mutations, nucleotide sequences were translated into the corresponding protein sequence by using the EMBOSS transeq software.

Selected reference sequences for concerned *Echinococcus* genotypes were those listed in alignments of polymorphic sites, with their GenBank entries (Figs 2, 4, 5, 7, 8). For *cox1* and *nad1* genes, sequences defined by Bowles et al. (1992) and Bowles and McManus (1993) were taken as reference ones, with corresponding accession numbers referenced to these studies. The only exception was associated with G1 genotype of *E. granulosus* s.s., for which respective GenBank entry (GbR M84661) differed by two nucleotides from the published sequence, and therefore was replaced by sequence with GbR DQ062857 (originated from Sardinia, Italy). For *atp6*, a reference G1 sequence was that reported by Le et al. (2002) (corresponding GbR AF297617). For *rrnS* gene, G1, G6, G7 reference sequences were derived from a study of Dinkel et al. (2004), and G3 sequence was recently provided by Busi et al. (2007).

Results

Based on the data obtained from all examined genes of the 22 isolates, nine isolates (four from sheep, five from humans) were identified as bearing genotype G1, eight isolates (four from sheep, four from humans) as microvariants of G1 (four isolates with type G1A, three with G1B, one with G1C), one isolate (from sheep) had genotype G3, one isolate (from sheep) had an intermediate sequence between G1 and G3, and three isolates (two from sheep, one from human) were homologous to G7 (including one microvariant). An overview of host and geographic distribution of isolates, gene fragments, genotype allocations, and nucleotide substitutions in variants are presented in Table 2. Fig. 1 illustrates the geographical origin of the indicated genotypes. In the following, we present the detailed results obtained with individual gene fragments:

cox1 gene (366 bp)

Six of nine examined isolates (S1, S2, S3, S8, S10, H1) produced sequences fully corresponding with the G1 reference sequence (G1 ref). Two isolates (S4, H10) possessed the non-synonymous nucleotide substitution 56C/T (inducing substitution of alanine with valine) that conforms to G2 ref at this site. In the two remaining polymorphic G1–G3 sites, these isolates displayed bases defined for G1 genotype and differing from G2/G3 pair (this haplotype is herein referred to as G1A, Table 2). One isolate (S6) gave a sequence identical to G7 (differing in one base from G6). The alignments of variable sites and neighbor-joining tree for this locus are presented in Figs. 2, 3.

cox1 gene (789 bp)

To more thoroughly assess the genetic variation in the sensitive *cox1* marker, a longer 789-bp region of the same gene was examined in 11 additional isolates and two isolates (S1, S2) already analyzed for the 366-bp fragment. The studied fragment overlaps with the second half of the 789-bp region downstream, with additional regions of 420 bases located

Table 2 Genotypes of examined isolates

Isolate	Int. host	Origin	*cox1* (366bp)	*cox1* (789bp)	*atp6*	*nad1*	*rrnS*	Final genotype
S1	sheep	Izmir	G1	G1B (309C/T)	G1			G1 variant (G1B)
S2	sheep	Izmir	G1	G1	G1	G1A (282C/T)		G1
S3	sheep	Izmir	G1		G1	G1A (282C/T)		G1
S4	sheep	Izmir	G1A (56C/T)		G1			G1 variant (G1A)
S5	sheep	Izmir		G1/G3 (486C/T=66C/T[a], 366A/G, 399T/C, 648A/G)	G1			G1/G3
S6	sheep	Izmir	G7				G7	G7
S7	sheep	Izmir				G7	G7	G7
S8	sheep	Menemen-Izmir	G1				G1	G1
S9	sheep	Bergama-Izmir		G3 (393C/T)	G1D (322G/A, 342T/C, 379G/A)			G3
S10	sheep	Manisa	G1				G1	G1
S11	sheep	Denizli		G1	G1C (379G/A)			G1 variant (G1C)
S12	sheep	Uşak		G1A (476C/T=56C/T[a])	G1			G1 variant (G1A)
H1	human	Izmir	G1				G1	G1
H2	human	Izmir		G1	G1			G1
H3	human	Izmir		G1A (476C/T=56C/T[a])	G1A (267A/G, 483T/C)			G1 variant (G1A)
H4	human	Izmir		G1	G1			G1
H5	human	Izmir		G1	G1			G1
H6	human	Izmir		G1B (309C/T)	G1			G1 variant (G1B)
H7	human	Izmir		G1				G1
H8	human	Bergama-Izmir		G1B (309C/T)	G1B (422T/C)			G1 variant (G1B)
H9	human	Selçuk-Izmir				G7A (168T/C)	G7	G7 variant (G7A)
H10	human	Manisa	G1A (56C/T)				G1	G1 variant (G1A)

[a] Corresponding mutation in short *cox1* fragment

upstream from the 5′-end and three bases extending the 3′-end compared with the former gene portion.

Five sequence types corresponding to G1 and G3 genotypes were detected at this locus. Six isolates (S2, S11, H2, H4, H5, H7) had sequences completely homologous to G1 ref. Two isolates (S12, H3) presented a C/T exchange at position 476 (which corresponds to position 56 in the 366-bp fragment) and were therefore allocated to the G1A variant. In three isolates (S1, H6, H8) C/T exchange at position 309 was detected (referred to as G1B). One isolate (S9) showed a homology with G3 structure. Apart from bases related to G3 pattern in polymorphic G1-3 sites, the S9 also produced specific 393C/T mutation. One isolate (S5) had an intermediate sequence between G1 and G3 reference patterns. The 486C/T substitution (analogous to 66CT in short *cox1*) seen in S5 is typical for G2/G3 pair, whereas the 366A/G, 399 T/C, 648A/G substitutions were not reported before. Among sequences deposited in GenBank database for long *cox1*, only one Chinese isolate (reference AY386206) expressed similar G1/G3 intermediate structure (with one additional mutation). Of eight recorded nucleotide exchanges in the long *cox1* fragment, only two substitutions produce amino acid changes, specifically, 476C/T (alanine/valine), found in S12 and H3 (G1A), and 677 T/C (valine/alanine), in S9 (G3). The sequence alignments for this gene are given in Fig. 4.

atp6 gene (513 bp)

Five haplotypes attributable to *E. granulosus* s.s. were found in 14 examined samples in this gene. Of these, 10 isolates (S1-S5, S12, H2, H4-H6) were completely identical to G1 ref. Two polymorphisms (267A/G, 483 T/C), not reported before, were detected in the isolate H3 (variant G1A). One specific mutation (422 T/C) was present in H8 isolate (variant G1B). Substitution 379G/A in S11 isolate (referred to as G1C) had previously been found in Australia (GbR AB027558). The S9 isolate (variant G1D) presented two mutations (342 T/C, 379G/A), which were previously detected in isolates from India (GbRs EF394905, EF394907, EF394913, EF423799) and China, Qinghai province (EU072118, EU072117), but produced also a third mutation 322G/A.

As no reference sequences were defined for G2 and G3 genotypes for *atp6*, it was not possible to derive association

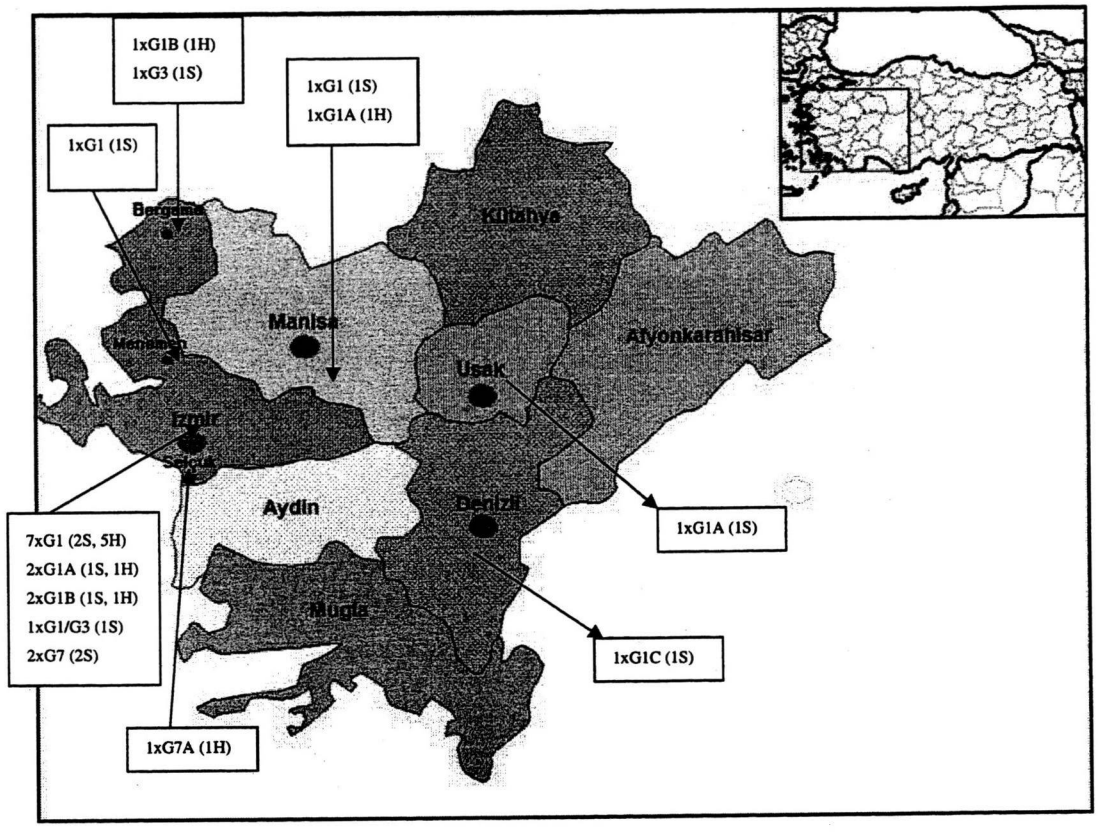

Fig. 1 Geographical origin of detected genotypes

of these mutations with the G2/G3 pair. The nucleotide substitution 379G/A observed in the two samples is non-synonymous (alanine/threonine), which was also the case of 322G/A (valine/threonine) and 422 T/C (valine/alanine) exchanges. Only three of six detected mutations were thus synonymous in this gene region.

The alignments and dendrogram for *atp6* are given in Figs. 5 and 6. Bootstrap values in branches of main nodes (Fig. 6) were relatively small (=50) due to presence of single identified species in samples scored at the locus.

```
                      1 1111111122 2222222333 3333
                 113455560 1124457923 4456789011 2456
                3356316765 1434794587 6877637058 1850
G1ref-DQ062857  TAATGTCGCG CGTGGGCGAG GGTGAGGGAT GAAG
G2ref-M84662    ......T.T. .......... ..C....... ....
G3ref-M84663    ........T. .......... ..C....... ....
G6ref-M84666    .GTGTGTTTA TTAAATTTGA TACTGATACC TGGA
G7ref-M84667    C..........  .......... .......... ....
N=6 (5xS, 1xH)  ..........  .......... .......... ....
N=2 (1xS, 1xH)  ......T...  .......... .......... ....
N=1 (1xS)       CGTGTGTTTA TTAAATTTGA TACTGATACC TGGA
```

Fig. 2 Alignment of variable sites in the partial *cox1* sequences (366 bp) with available sequences of related reference genotypes. *N* Number of isolates detected for each variant, *S* sheep host, *H* human host. *Dots* indicate identity with reference sequences

nad1 gene (471 bp)

Both isolates identified as G1 (S2, S3) in this gene fragment displayed 282C/T mutation (synonymous), which was formerly defined for G2 ref and G3 ref. However, nucleo-

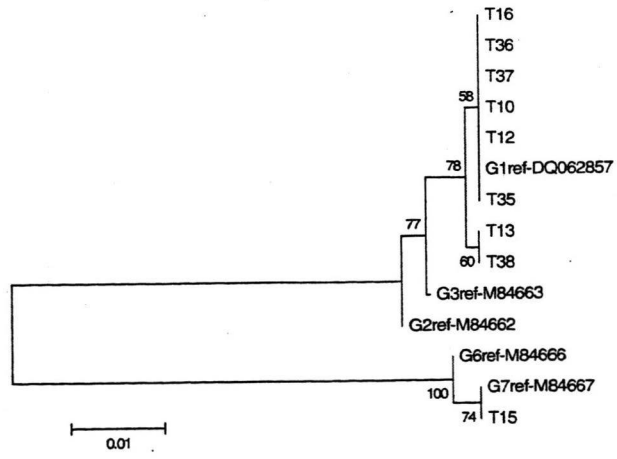

Fig. 3 Dendrogram obtained for *Echinococcus* isolates/genotypes by neighbor-joining analysis at *cox1* locus (366 bp). Numbers at the nodes indicate percentage bootstrap support obtained in 1,000 replications

```
                    33334466
                    06997847
                    96396687
G1ref-AB033407      CACTCCAT
N=6 (4xH, 2xS)      ........
N=3 (2xH, 1xS)      T.......
N=2 (1xH, 1xS)      ....T...
N=1 (1xS)           ..T..T.C
N=1 (1xS)           .G.C.TG.
```

Fig. 4 Alignment of variable sites in the partial *cox1* sequences (789 bp) with available sequences of related reference genotype. *N* Number of isolates detected for each variant, *S* sheep host, *H* human host. *Dots* indicate identity with the reference sequence

tides at the two other polymorphic sites corresponded to the G1 ref sequence. Two isolates (S7, H9) revealed sequences associated with G7 genotype. S7 was entirely homologous to G7 ref, whereas H9 had a synonymous T/C substitution at position 168. The alignments of the polymorphic sites for this locus are given in Fig. 7.

rrnS gene (295 bp)

Sequences in a portion of *rrnS*, which allows for a consistent differentiation of the genotype pairs G1/G3 and G6/G7, gave monomorphic patterns within the two detected variants. Amongst seven isolates resolved in this gene, four isolates (S8, S10, H1, H10) possessed G1 sequences. The identification of G7 in three isolates (S6, S7, H9) was based on the C/T exchange between G6 and G7 at position 165. The sequence alignments for the locus are given in Fig. 8.

Discussion

The present study provides further evidence for the predominant presence of *Echinococcus granulosus* sensu stricto (G1–G3 cluster) in Turkey, and its role as the principal agent of human CE.

A major focus of this study was the characterization of intraspecific variants in the mitochondrial genome of this taxon as a contribution to ongoing discussions on the validity of the 'strains' G1, G2, and G3 (Busi et al. 2007). A large set of mitochondrial sequence data had been accumulated for

```
                    2333 44
                    6247 28
                    7229 23
G1ref-AF297617      AGTG TT
N=10 (6xS, 4xH)     .... ..
N=1 (1xS)           .ACA ..
N=1 (1xS)           ...A ..
N=1 (1xH)           .... C.
N=1 (1xH)           G... .C
```

Fig. 5 Alignment of variable sites in the partial *atp6* sequences (513 bp) with available sequences of related reference genotype. *N* Number of isolates detected for each variant, *S* sheep host, *H* human host. *Dots* indicate identity with the reference sequence

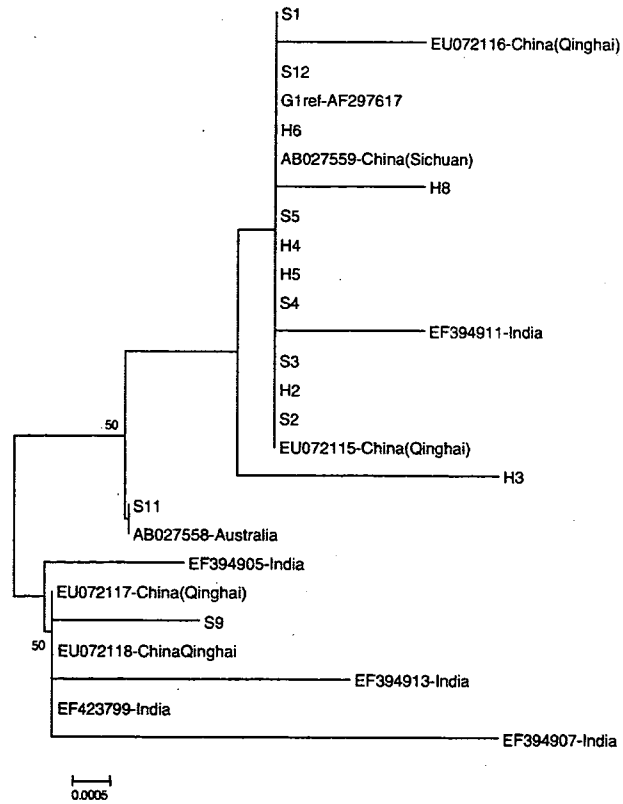

Fig. 6 Dendrogram obtained for *Echinococcus* isolates (including data from isolates deposited in GenBank) by neighbor-joining analysis at *atp6* locus (513 bp). Numbers at the nodes indicate percentage bootstrap support obtained in 1,000 replications

this genotype cluster over the last two decades. Based on amplification of a 366-bp fragment of the *cox1* gene, Bowles et al. (1992) defined the genotypes G1 ("common sheep strain") and G3 ("buffalo strain") by differences in 3 bp at positions 56, 66 and 257, and G2 ("Tasmanian sheep strain") by a difference at position 56 from G1 and G3. In our study, four isolates from three Turkish provinces produced a haplotype (G1A) bearing the substitution 56C/T identical to the G2 ref sequence, whereas in two other polymorphic sites nucleotides matched with the G1 sequence. This haplotype is widespread: according to GenBank® data this pattern has been found in Morocco (GbR EF367285 and six additional isolates), China (DQ356877), Austria (AJ508019) and in three previously examined isolates from Turkey (EU178104, EF689726, AJ508012). In addition, this variant was recorded by M'Rad et al. (2005) who found the 56C/T substitution in seven of 37 Tunisian G1 isolates. The haplotype with the reference G2 structure has been less often recorded, so far from Morocco (EF367294, EF367293), India (DQ269944), in the two Tasmanian reference isolates (M84662) and in three isolates from Argentina (Rosenzvit et al. 1999). The reference G3 sequence was found in 11 isolates from 6 countries in GenBank. The most abundant

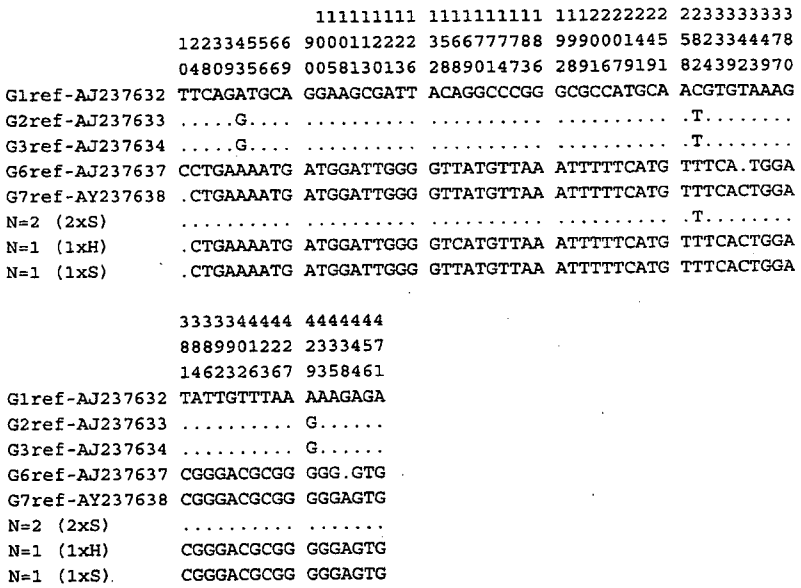

Fig. 7 Alignment of variable sites in the partial *nad1* sequences (471 bp) with available sequences of related reference genotypes. *N* Number of isolates detected for each variant, *S* sheep host, *H* human host. *Dots* indicate identity with reference sequences

genetic type within the G1–G3 cluster in this database constitutes the sequence ascribed to the reference G1 structure (32 isolates from eight countries carrying this pattern). Polymorphism at position 56 (cytosine/thymine) in *cox1* has not consistently correlated with the strain identity when several gene markers were used (Busi et al. 2007; Rinaldi et al. 2008a). Considering also the above occurrence of sequence types, this polymorphic pattern seems to represent only intrastrain variability of G3 or G1, with the exclusion of G2 as a separate genotype. Similar conclusions were recently drawn by Vural et al. (2008) following data gathered from widely distributed sites in Turkey.

From three proposed discriminative positions in *cox1* by Bowles et al. (1992), only position 257 appears to be consistently diagnostic for G1 and G3 genotypes due to paucity of data with mixed G1/G3 structure derived from variability at this site. Specifically, no sequence with CCC bases located at three differentiating positions and only one GenBank entry with TCC bases were so far documented in *E. granulosus* s.s. The single case of isolate bearing the TCC structure (Morocco, GbR EF367260) could have

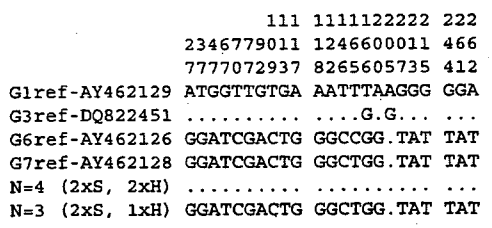

Fig. 8 Alignment of variable sites in the partial *rrnS* sequences (295 bp) with available sequences of related reference genotypes. *N* Number of isolates detected for each variant, *S* sheep host, *H* human host. *Dots* indicate identity with reference sequences

arisen by introgressive hybridization between G1 and G3 genotypes which might occur if reproductive isolation has not been completed, although such a process is rare in mitochondrial genomes with their predominant clonal heredity (Avise 2000). The diagnostic relevance of position 66 seems to be weaker, considering the existence of several mixed G1/G3 haplotypes with compromised structure at this site (corresponding CTT nucleotides at differentiating sites recorded in six isolates from two countries within GenBank and one sheep isolate in our study).

In the *nad1* gene (471 bp), Bowles and McManus (1993) differentiated G1 from G2/G3 group by differences at positions 43, 282, and 429. G2 and G3 genotypes were not discernible in this gene fragment. In our study, both G1 isolates had a single exchange (282C/T) which was defined as characteristic for G2 and G3 by the above authors, but in the remaining polymorphic G1–G3 sites nucleotides matched with G1. The same haplotype was elsewhere found in Morocco (GbR EF367308 and 13 additional isolates), India (EF179166), China (Y572546 plus additional 8 isolates), Algeria (AF408688), Greece (DQ856470), Italy (DQ154008), Austria (AJ508081 AJ508070), and is thus globally of markedly higher abundance (29 isolates from seven countries) compared to the reference G1 haplotype (only two references in GenBank, referring to the same source of Bowles and McManus 1993). Therefore, the 282C/T nucleotide substitution seems to be associated with the G1 microvariant (and more likely even with the archetypal G1 structure) rather than with the G2/G3 forms. The haplotype defined for G2/G3 by Bowles and McManus (1993) was found in 11 isolates from four countries (India, Morocco, Greece, Romania) in GenBank database. Unlike this, no report was so far provided about the mixed G1/G3 structure inferred from variations at

positions 43 and 429 (i.e., under the simultaneous occurrence of ATG, ACG, GCA, GTA, GCG bases in variable sites); nucleotides at these sites are entirely homologous to either G1 or G3. These positions thus appear to be readily diagnostic for both genotypes.

The G2 genotype as previously defined cannot be distinguished from G3 by sensitive *rrnS* and *nad1* gene markers, which further diminishes the justification of G2 as being a separate genetic unit. We thus propose to allocate isolates with this polymorphism to either G3 or G1, depending on variations at position 257 (T/C) in the 366-bp *cox1* region and positions 43 (A/G) and 429 (A/G) in the 471-bp *nad1* region.

Genetic data gathered in this survey are rather fragmented as they were obtained consecutively in different laboratories and DNA of some isolates was not available in all stages of the study. However, the final strain classification was reliable as sequences of every sample were attained for at least two genetic markers. The putative ancestral G1 haplotype and derived haplotypes detected in the study (G1A, G1B, G1C) were distributed across the territory of western Turkey without apparent clustering. Of ten human samples infected with CE, nine samples produced patterns consistent with the G1 genotype and its microvariants, thus emphasizing the importance of the common sheep strain for public health. Among 22 isolates, only one was classified as G3 and one had an intermediate G1/G3 structure (both were sheep isolates from Izmir province). A similar pattern was recorded in the study of Vural et al. (2008) where only five isolates (4.46%) from eastern Turkey were classified as G3. Originally described from India (Bowles et al. 1992), this genotype seems to be widespread in the Mediterranean region. The G3 genotype was so far recorded in Italy (Capuano et al. 2006; Busi et al. 2007; Casulli et al. 2008; Rinaldi et al. 2008a, b), Romania (Bart et al. 2006), Greece (Varcasia et al. 2007), and Morocco (Azlaf and Dakkak 2006).

In conclusion, G1 and G3 are well-definable genotypic groups within *E. granulosus* s.s., although intermediate sequences do occasionally occur with some markers. Awaiting further data, it remains to be shown if this intraspecific variation is in any way correlated with differences in host specificity or pathogenicity, specifically towards its manifestation in humans.

For the first time, we report the presence of the pig strain (G7) of *E. canadensis* in Turkey. This form is well established and frequent in a contiguous area of eastern-central Europe that includes the territories of Poland, Slovakia, Ukraine and parts of eastern Germany (Kedra et al. 1999; Šnábel et al. 2000; Tkach et al 2002; Turčeková et al. 2003; Dinkel et al. 2006). It was also reported from isolated European foci of Spain (Gonzales et al. 2002), Italy (Sardinia) (Varcasia et al. 2006), Romania (Bart et al. 2006), Greece (Varcasia et al. 2007) and Lithuania (Bruzinskaite et al. 2005). Elsewhere, it has been recorded from Armenia (Gevorgyan et al. 2006), Argentina (Rosenzvit et al. 1999; Kamenetzky et al. 2002) and Mexico (Cruz-Reyes et al. 2007; Villalobos et al. 2007). In the current survey, three isolates derived from two sheep and one human from the southern part of Izmir province were genotyped as G7. As sheep are unusual hosts for this strain, which predominantly uses pigs as intermediate hosts, and considering the small numbers of domestic pigs in this mainly Muslim country, the transmission pattern in Turkey is not yet identified. Goats may be significant hosts, as they are frequently infected with unidentified CE in Turkey and, in contrast to sheep, are known to be suitable hosts for G7 in Spain and Greece (Daniel-Mwambete et al. 2004; Varcasia et al. 2007). In addition, the affinity of the closely related G6 genotype to this ruminant has also been demonstrated in Argentina and Kenya (Haag et al. 2004; Dinkel et al. 2004). As G7 is known to occur in neighboring Armenia and Greece, its introduction into Turkey via livestock trade appears feasible. Also, wild boar can be involved in G7 transmission through sylvatic environment, as was confirmed in Ukraine, Spain, and Germany (Kedra et al. 2000; Gonzales et al. 2002; Dinkel et al. 2006).

Recently, meat from wild boar and illegally kept backyard pigs was indicated as the likely source of a large trichinellosis outbreak in Izmir in 2004 with 418 affected people (Akkoc et al. 2009). Following that outbreak, strict regulations were issued at the national level for sale of minced meat or meat preparation, which was coupled with disclosure of many illegal pig farms around Izmir. The single human G7 case found in the study (site Selçuk, Izmir) had undergone surgery in December 2003, and in the same year the material from the two G7-infected sheep was collected. Therefore, improved market and slaughter surveillance might be the reason why no G7 was found in our samples collected since 2004 as well as in recent genetic studies on *Echinococcus* in Turkey (Utuk et al. 2008; Vural et al. 2008). Despite that humans seem to be poorly susceptible to infection with G7 even compared with related G6 genotype (Thompson 2008), the human case in the present study adds evidence to its infectivity under certain conditions.

The occurrence of the pig strain in Turkey has to be taken into consideration when designing hydatid control measures. The shorter prepatency period of G7 in dogs (on average 34 days) compared with G1 (45 days) requires a different drug treatment schedule for dogs to break the transmission cycle (McManus et al. 2003). The presence of G7 in western Asia also suggests that a sole use of PCR-RFLP assay, employed, e.g., in a study of Ahmadi and Dalimi (2006) describing G6 in Iran, is not sufficient for strain diagnosis as it does not distinguish between the

closely related G6 and G7 genotypes without DNA sequence analyses.

Interestingly, a G1 variant seemingly adapted to pig–dog transmission was found in Bulgaria in a study of Breyer et al. (2004). Unlike *nad1* sequences exhibiting a 100% homology with G1 in isolates from cattle, sheep, jackal, wolf, all six pig isolates originating from the same area (Stara Zagora region) possessed a 376C/T substitution. The identical T base is present in G7, and a possible adaptive function of this mutation to survival in the pig is open to further consideration.

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Short communication

Molecular identification of *Echinococcus* isolates from Peru

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ABSTRACT

Genetic variations in tapeworms causing cystic echinococcosis in Peru were investigated. Seventy one larval isolates collected from different intermediate hosts and geographic regions were identified by the DNA sequencing of genes for mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor 1 alpha (*ef1a*). The G7 genotype (*E. canadensis* pig strain) was found for the first time in pigs reared in the city of Lima. *Echinococcus granulosus* sensu stricto (sheep strain or G1) was the most prevalent in human patients, sheep, and cattle and the G6 genotype (*E. canadensis* camel strain) was found in goats and in one human patient. These findings may inform prevention strategies and control programs against echinococcosis in Peru.

Cystic echinococcosis (CE) is caused by the larval stage of tapeworms belonging to the cryptic species complex of *Echinococcus granulosus* sensu lato. CE occurs in sheep rearing regions of Peru where it is considered a major public health and economic problem [1]. Surveys in endemic villages have shown human infection prevalences ranging from 5.5–9.3% [1,2]. Molecular studies using mitochondrial DNA sequences have identified 10 distinct genotypes (G1–10) within *E. granulosus* sensu lato [3,4]. These include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camel strain (G6), two pig strain (G7 and G9) and cervid strains (G8 and G10). Because the taxon *E. granulosus* sensu lato is paraphyletic, the following taxonomic revision has been made: *E. granulosus* sensu stricto (G1–3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6–G10) [5]. However, some controversy continues to exist regarding the taxonomical status of *E. canadensis*. Lavikainen et al. [4] and Moks et al. [6] have emphasized the close relationship between *E. ortleppi* (G5) and *E. canadensis* (G6–G10) whereas Thompson [7] suggests G6 and G7 could represent a species of its own, *E. intermedius*, distinct from *E. canadensis* (G8 and G10). *E. granulosus* sensu stricto is most commonly associated with human infections worldwide. Knowledge of the frequency, distribution in different host species and geographic distribution of these genotypes is important for development of control strategies because some of them have different growth and development characteristics. This study describes a survey of *Echinococcus* isolates from different hosts and regions in Peru.

Hydatid cysts were obtained from different animal hosts slaughtered at the Yerbateros abattoir in the city of Lima (Table 1). In addition, human hydatid cysts excised from human patients who had surgery for CE at the Department of Thoracic Surgery at Hospital Nacional Hipolito Unanue in Lima were also examined. Ethical approval was obtained from Ethical Committees of Asociación Benéfica PRISMA and Hospital Nacional Hipólito Unanue in Lima, Perú. Animal and human samples were collected during October 2006–January 2007. An *Echinococcus* isolate was defined as protoscoleces obtained from a single hydatid cyst. The genomic DNA of each isolate was prepared from 70% ethanol-preserved protoscoleces by using DNeasy blood and tissue kit (Qiagen, Germany), and used as a template for polymerase chain reaction (PCR). Regions targeted for PCR were a mitochondrial gene for cytochrome *c* oxidase subunit 1 (*cox1*) and a nuclear gene for elongation factor 1 alpha (*ef1a*). The PCR was carried out by using a published primer set for *cox1* [8] or an original primer set for *ef1a* (5′-AATGGATGCAG TTGATTACAGTGA-3′ and 5′-GCAGGGTTAGTCTCCTTGA CCTGA-3′). Thermal reactions were performed for 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. Each PCR product was directly sequenced as reported previously [5]. The nucleotide lengths determined were 789 bases in *cox1* and 656 bases in *ef1a*. The phylogenetic tree of *cox1* genotypes was constructed by neighbour-joining method [9].

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Table 1
Geographic origin and hosts of Peruvian isolates of *Echinococcus* identified by the DNA sequencing of mitochondrial *cox1* gene.

Geographic origin	*E. granulosus* sensu stricto						*E. Canadensis*					Total
	Sh	Ca	Pi	Go	Hu	Total	G6			G7		
							Go	Hu	Total	Pi	Total	
Cajamarca	–	7	–	–	–	7	–	–	0	–	0	7
Ancash	–	1	–	–	–	1	–	–	0	–	0	1
Ayacucho	2	4	–	–	–	6	–	–	0	–	0	6
Lima	13	2	4	1	1	21	–	1	1	8	8	30
Junín	–	–	–	–	1	1	–	–	0	–	0	1
Huancavelica	–	–	–	–	2	2	–	–	0	–	0	2
Ica	17	–	–	–	–	17	2	–	2	–	0	19
Moquegua	–	–	–	–	–	0	3	–	3	–	0	3
Puno	–	2	–	–	–	2	–	–	0	–	0	2
Total	32	16	4	1	4	57	5	1	6	8	8	71

Hosts are sheep (Sh), cattle (Ca), pigs (Pi), goats (Go), and humans (Hu).

A total of 80 larval *Echinococcus* isolates were obtained from 32 sheep, 16 cattle, 21 pigs, 6 goats, and 5 humans. The PCR amplification of *cox1* was successful in 71 isolates. The negative results of 9 isolates from pigs were probably due to the low concentration or the degeneration of DNA. Table 1 shows the geographic origins and hosts of 71 *Echinococcus* isolates identified by *cox1* genotyping. Fifty seven (80%) isolates were identified as *E. granulosus* sensu stricto, which included 5 haplotypes (designated as *Egra*-A to *Egra*-E). Six (9%) isolates corresponded to the G6 genotype of *E. canadensis* with 2 haplotypes (*Ecan*-A and *Ecan*-B), and 8 (11%) isolates to the G7 genotype of *E. canadensis* with 1 haplotype (*Ecan*-C). The phylogenetic relationships of these haplotypes were illustrated in Fig. 1. The mitochondrial haplotypes found in this study were deposited into DDBJ/EMBL/GenBank databases under the accession numbers AB458672-5 and AB470527 (*Egra*-A to *Egra*-E) and AB458676-8 (*Ecan*-A to *Ecan*-C).

Two species of *Echinococcus* were found from humans. As shown in Table 1, 4 of 5 patients were infected with *E. granulosus* sensu stricto, and the remaining one with *E. canadensis* (G6 genotype). All patients were males with an age range 10 to 28 years (mean 20 years). Two patients had a non-complicated lung cyst and 3 had a complicated lung cyst. No distinguishing clinical features were observed in the patient infected with the G6 genotype. All 32 sheep and 16 cattle were infected with *E. granulosus* sensu stricto. The dominant parasite in 6 goats was *E. canadensis* (G6 genotype), although one isolate was identified as *E. granulosus* sensu stricto (G1 genotype). In pigs, 8 of 12 isolates were identified as *E. canadensis* (G7 genotype), while 4 isolates as *E. granulosus* sensu stricto. Seven of the 12 isolates were originated from liver, 1 from lung, and 4 from kidney. The liver-specific tropism was found in pigs infected with the G7 genotype. In the PCR amplification of nuclear *ef1a*, positive results were obtained from 67 isolates. The PCR positive rate of nuclear *ef1a* was smaller than that of mitochondrial *cox1*, probably due to the low copy number of the nuclear gene. The DNA sequencing of *ef1a* proved 55 isolates to be *E. granulosus* sensu stricto and 12 isolates to be *E. canadensis*. The G6 and G7 genotypes of *E. canadensis* showed the identical sequences of *ef1a*. Mitochondrial introgression was not observed in all isolates examined.

We identified the G7 genotype for the first time in pig isolates from Peru. A previous study in Peru reported the presence of the G1 and G6 genotypes in human isolates [10], however in our study we examined a larger number of hosts from very diverse geographic regions of Peru and this allowed us to identify the G7 genotype. In our study, all pig isolates originated from districts surrounding the city of Lima, and this finding alone is cause of concern as it demonstrates *Echinococcus* transmission in the city of Lima considered to be a low endemic area for this cestode. In most areas of Peru, pigs are raised along with other livestock and are allowed to roam freely in the fields where they can have easy access to feces of dogs infected with *Echinococcus* spp. In some highly endemic areas, the prevalence of swine echinococcosis can be as high as 83% [11]. It is important to determine if the G7 genotype is as prevalent in other *Echinococcus* endemic areas of Peru as it is in Lima pigs or if Lima pigs present a unique situation that favours the occurrence of this genotype alone given the absence of other intermediate hosts in Lima. The G7 genotype has been previously described in pig isolates from Argentina [12–14] and Mexico [15] but thus far it has not been isolated in humans in South America. An interesting finding was the predominance of liver hydatid cysts among pigs infected with the G7 genotype which is in agreement with previous findings that report a predominant liver localization for cysts of the G7 genotype [16]. Although it would appear that the pig strain is poorly infective to humans, further studies should help define the distribution of the G7 genotype in Peru and its presence in the human population.

We found one Peruvian patient to be infected with the G6 genotype, and we did note that most of the G6 isolates occurred in goats, suggesting this species can be one of the reservoirs for this genotype. The other Latin America country where the G6 genotype has been described is Argentina [12]. Future studies with human and lower animal isolates from different geographic regions of Peru should further elucidate the frequency of the G6 genotype in human patients and other intermediate hosts. The finding of the G6 and G7 genotypes in Peru has important implications for public health. In dogs, for example, both genotypes develop into adults more quickly than the more common *E. granulosus* sensu stricto [16,17]. Intervention

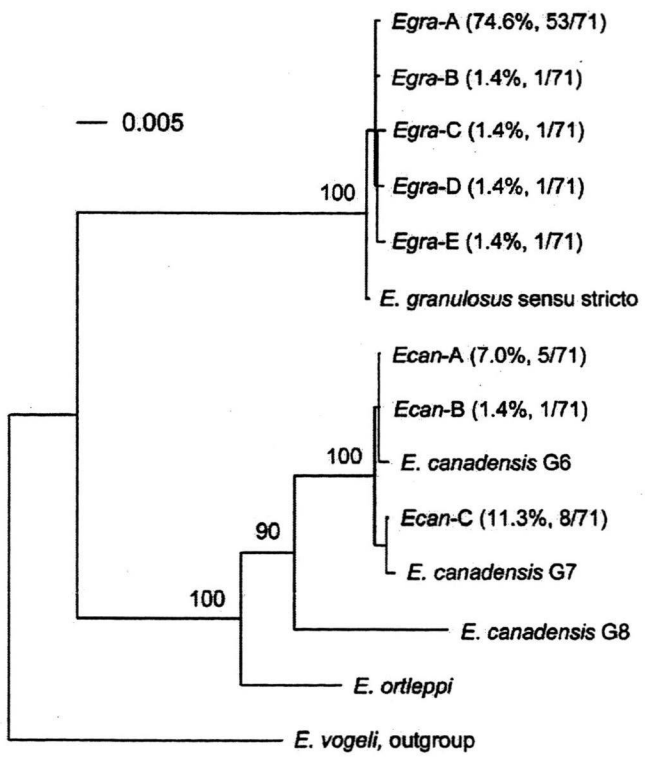

Fig. 1. The neighbor-joining tree of Peruvian *Echinococcus* spp. constructed by using partial DNA sequences of mitochondrial *cox1* gene. *Egra*-A to *Egra*-E and *Ecan*-A to *Ecan*-C are individual haplotypes found in Peruvian isolates. The frequencies of the haplotypes are shown in parenthesis. The tree includes the representative sequences of *E. granulosus* sensu stricto (database accession no. AF297617), the G6 genotype of *E. canadensis* (AB208063), the G7 genotype of *E. canadensis* (AB235847), the G8 genotype of *E. canadensis* (AB235848), *E. ortleppi* (AB235846) and *E. vogeli* (AB208546). Bootstrap values (%) are shown above branches. The scale bar represents the estimated number of nucleotide substitutions per site.

programs based on periodic anthelminthic treatment of canine hosts infected with the G6 and G7 genotypes may require shorter treatment intervals than those directed at other, more slowly maturing strains of *Echinococcus*. This may mean greater expenditures for purchasing anthelmintics which could add financial constraint to control programs in developing countries.

In summary, the G7 genotype of *E. canadensis* (pig strain) has been found for the first time in Peruvian pigs from the city of Lima, demonstrating the involvement of *E. canadensis* in the urban transmission cycle of *Echinococcus*. *E. granulosus* sensu stricto (sheep strain) is predominant in animal and human hosts in Peru, but the G6 genotype of *E. canadensis* (camel strain) is also present in goats and can cause human infection.

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Close Relationship between Clinical Regression and Specific Serology in the Follow-up of Patients with Alveolar Echinococcosis in Different Clinical Stages

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Abstract. We compared four enzyme-linked immunosorbent assays (ELISAs) with 172 serum samples from 28 patients with alveolar echinococcosis in different clinical stages according to the World Health Organization–PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, M = metastasis) staging system. The sequential antibody responses against $Em2^{plus}$, Em10, and Em18 antigens, and a crude antigen extract were measured in cohorts with resected and unresected lesions. Antibody levels in all assays correlated with the PNM stage before treatment, and the highest correlation was shown for the Em18 assay. The PNM stage did not influence the antibody kinetics, but changes in antibody levels depended on the treatment. In patients after curative surgery, seroreversion in the $Em2^{plus}$ ELISA indicated successful resection of lesions in more patients than any other assay, irrespective of the clinical stage. There were no significant differences in the time before assays that use recombinant or purified antigens became unreactive. Antibodies directed against crude antigens were detectable longer than other antibodies in all patient cohorts and stages.

INTRODUCTION

Alveolar echinococcosis (AE) is an important parasitic zoonosis of the northern hemisphere. It is caused by the larval stage (metacestode) of the fox tapeworm *Echinococcus multilocularis*. The disease is characterized by infiltrative growth of the metacestode in the liver of suitable natural intermediate hosts and accidentally in humans. Because metastasis may occur, the biological characteristics of the metacestode are similar to a malignant tumor. Radical resection of parasitic lesions is the preferred treatment,[1] but the timely detection of recurrence after surgery is difficult. However, most patients are inoperable at the time of diagnosis.[2,3] In such cases, the determination of disease activity (i.e., growth of parasitic lesions) and response to antiparasitic chemotherapy is important. Imaging tools, such as ultrasound and computed tomography are currently the basic monitoring techniques.[4] Enlargement of lesions is slow and demonstration of progressive AE by imaging requires a period of several months. Disease progression can therefore only be evaluated retrospectively by imaging.[2] ^{18}F-fluorodeoxyglucose positron-emission tomography (FDG-PET) has also been used to monitor disease activity. However, the results of this real-time investigation are controversial.[2,4–6] Serologic analysis with affinity-purified and recombinant antigens has recently shown a promising correlation with disease activity.[7,8]

In this study, we tested four enzyme-linked immunosorbent assay (ELISAs) in parallel with serum samples from 28 patients with resected or unresected parasitic lesions in different clinical stages of AE according to the World Health Organization (WHO)–PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, M = metastasis) system.[9] The sequential antibody responses against two recombinant antigens (Em10 and Em18), an affinity-purified antigen combined with a recombinant antigen ($Em2^{plus}$), and a crude antigen extract were measured and compared in 172 serum samples.

PATIENTS AND METHODS

Patients. All patients in this study attended the University Hospital and Medical Center in Ulm, Germany. A total of 28 patients with the history of hepatic AE and a follow-up period of 1.5–6.5 years were included in the study. The patients (age range = 17–74 years, mean age = 51.2 years, sex ratio [M:F] = 0.4:1) were in different clinical WHO-PNM stages of the disease. All patients had acquired AE in Germany and received benzimidazole therapy. Twelve patients had curatively resected lesions, 2 had recurrences after surgery, 1 had a palliative resection only, 11 had unresectable lesions but stable disease, and 2 had apparently dead, fully calcified lesions (Table 1). Serum samples were tested at the Institute of Hygiene and Microbiology, University of Würzburg, Germany (crude larval antigen-, $Em2^{plus}$- and Em10-ELISA), and at the Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan (Em18-ELISA) in a blind test. Classification of curative resection, stable disease, progressive disease, or presence of an apparently dead, fully calcified lesion was established by magnetic resonance imaging on the basis of lesion size and morphology at the respective follow-up intervals. The study was reviewed and approved by the ethics committee of the University of Ulm.

Methods. For the crude larval antigen ELISA, *E. multilocularis* metacestode tissue harvested from the peritoneal cavity of Mongolian jirds was mechanically homogenized and centrifuged. The supernatant was used to coat microtiter plates at a concentration of 2 ng/μL. Patient serum samples were tested at a dilution of 1:300 after pre-absorption of the wells with 2% skimmed milk (Merck, Darmstadt, Germany). Serum antibodies bound to echinococcal antigens were detected by secondary peroxidase-conjugated anti-human IgG antibodies (Dako, Glostrup, Denmark) using 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) (ABTS) (Roche, Mannheim, Germany) as chromogenic substrate. Absorbance was measured after 60 minutes at 410 nm with a reference wavelength of 490 nm. For

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TABLE 1
Characteristics of 28 patients with alveolar echinococcosis included in the study

Patient no.	Stage	PNM* code	Status†	Age, years‡	Sex	Follow-up duration, years
1	I	P1N0M0	Curative resection	62	F	5.5
2	I	P1N0M0	Curative resection	24	F	5
3	I	P1N0M0	Curative resection	22	F	4
4	I	P1N0M0	Curative resection	33	F	2
5	I	P1N0M0	Apparently dead, fully calcified lesion	58	M	4
6	I	P1N0M0	Unresectable, stable disease	61	M	3.5
7	II	P2N0M0	Curative resection	38	F	3
8	II	P2N0M0	Unresectable, stable disease	71	M	6
9	II	P2N0M0	Unresectable, stable disease	68	F	5.5
10	II	P2N0M0	Unresectable, stable disease	59	F	6.5
11	II	P2N0M0	Unresectable, stable disease	60	F	6
12	IIIa	P3N0M0	Curative resection	25	F	4.5
13	IIIa	P3N0M0	Curative resection	62	M	5.5
14	IIIa	P3N0M0	Recurrence after resection	17	F	3.5
15	IIIa	P3N0M0	Unresectable, stable disease	69	F	6
16	IIIa	P3N0M0	Unresectable, stable disease	39	F	4
17	IIIa	P3N0M0	Apparently dead, fully calcified lesion	57	F	6
18	IIIb	P3N1M0	Progression after palliative resection	32	M	6
19	IIIb	P4N0M0	Unresectable, stable disease	60	F	5
20	IIIb	P3N1M0	Unresectable, stable disease	49	F	5.5
21	IIIb	P2N1M0	Recurrence after resection	50	M	5
22	IIIb	P3N1M0	Unresectable, stable disease	74	F	1.5
23	IV	P4N1M0	Curative resection	56	F	6.5
24	IV	P4N1M0	Curative resection	30	M	3
25	IV	P4N1M0	Curative resection	72	F	3
26	IV	P4N1M0	Unresectable, stable disease	71	F	5.5
27	IV	P4N1M0	Curative resection	52	M	2.5
28	IV	P4N1M0	Curative resection	63	F	1.5

*PNM = P = parasitic mass in the liver; N = involvement of neighboring organs; M = metastasis.
†Assessed by imaging (apparently dead lesion, progressive disease and stable disease) or imaging and histologic analysis (curative resection).
‡Age when first blood sample was obtained.

the calculation of the cut-off, the mean value of the absorbance of 12 serum samples from healthy blood donors was added to 3 × the SD. The index of the individual serum sample was calculated by dividing the sample's absorbance by the cut-off.

The Em2^{plus} ELISA (Bordier Affinity Products, Crissier, Switzerland) was used according to the manufacturer's instructions. Indices for the Em2^{plus} ELISA were calculated by dividing the individual serum absorbance by the provided weak positive control serum (cut-off serum). For the Em10 ELISA, microtiter plates were coated with recombinant Em10 antigen[10] at a concentration of 10 ng/μL. Patient serum samples were tested as described for the larval antigen extract ELISA. For the Em18 ELISA, recombinant Em18 antigen[11] was used to coat microtiter plates at a concentration of 100 ng/well. Patient serum samples were tested at a dilution of 1:100 after pre-absorption of the wells with 1% casein in 20 mM Tris-HCl (pH 7.4)-150 mM NaCl buffer. Serum antibodies bound to echinococcal antigens were detected by secondary peroxidase-conjugated protein G (Zymed, South San Francisco, CA) using ABTS (Sigma) as chromogenic substrate. Absorbance was measured after 30 minutes at 405 nm with a reference wavelength of 630 nm. For the calculation of the cut-off, the mean value of the absorbance of 40 sera from healthy blood donors was added to 3 × the SD. The index of the individual serum sample was calculated by dividing the sample's absorbance by the cut-off.

Statistical analysis. Statistical analyses were done with the free software environment R for statistical computing. Non-parametric data were analyzed using Spearman's rank test for the correlation of the clinical stage and the ELISA index of the respective assays. Wilcoxon's rank sum test was used to examine differences in the time to negativity of the various assays. P values < 0.05 were regarded as significant.

RESULTS

The height of the ELISA index of all tests correlated weakly with the clinical PNM stage prior to treatment. The highest correlation was shown for the median values of the Em18 ELISA (Figure 1). The PNM stage *per se* did not influence the kinetics of the antibodies but changes of antibody levels strongly depended on the type of treatment the individual patient underwent in each stage (Figure 2A and B). In general, there were good agreements in the patterns of all assays tested.

In the cohort of 12 patients who underwent curative resection, antibody levels decreased rapidly after resection of the parasitic mass and the surrounding inflammatory tissue in all assays. Levels of antibodies decreased below the cut-off level of the respective assays (Figures 2A and 3A). In some patients, two or all three ELISAs, which used recombinant and/or purified antigens, became unreactive at the same time. The time elapse before antibodies became undetectable in the various assays is shown in Figure 4. More patients had a decrease in antibody levels below the cut-off when tested by the Em2^{plus} assay than by the Em10 assay or the Em18 assay (Figures 3A and 4). The time before antibodies became undetectable was significantly shorter in the Em^{2plus} assay than in the crude antigen ELISA (P = 0.013). No significant differences in the time to negativity could be demonstrated between

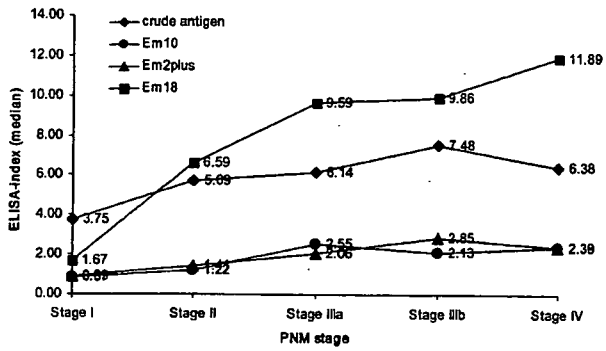

FIGURE 1. Correlation of PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, M = metastasis) stage and enzyme-linked immunosorbent assay (ELISA) index prior to treatment. Median index values of the respective assays per PNM stage are shown. An increase in median values of indices in parallel with clinical stage is present for all antigen preparations. The Em2^{plus} and Em10 ELISAs demonstrated the lowest median indices in all stages. The Em18 ELISA had the highest indices, except for stage I. No prominent changes of the Em2^{plus}, Em10, and Em18 indices are visible between stages IIIa and IIIb. Between stages IIIb and IV, only the Em18 index shows a marked increase. Spearman's rank correlation coefficients (ρ) for the Em^{2plus}, Em10, Em18, and crude antigen assay and the PNM stages were 0.421, 0.430, 0.483, and 0.319, respectively.

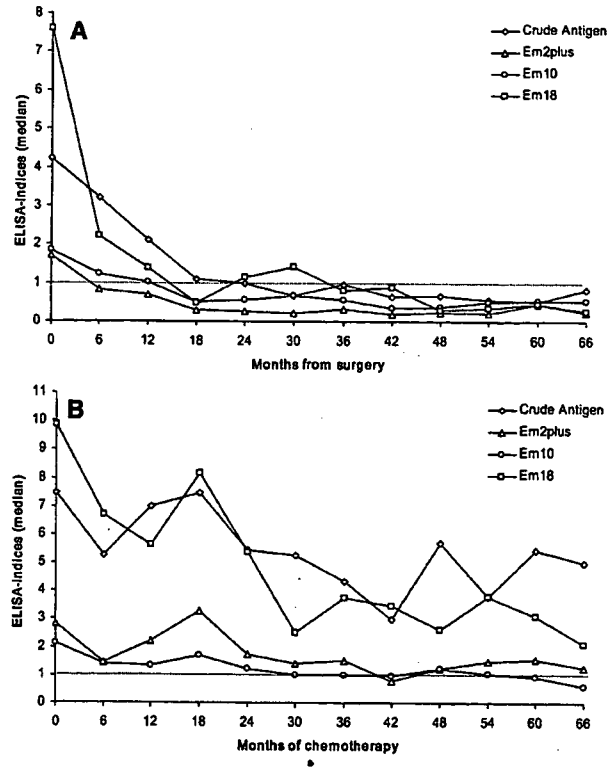

FIGURE 2. **A**, Antibody profiles in patients after curative resection. Median index values of all assays from 12 patients in different clinical stages are shown. A rapid decrease of all antibody indices is clearly visible. **B**, Antibody profiles in patients with stable disease and unresectable lesions. Median index values of all assays from 11 patients in different clinical stages are shown. A slow decrease in all antibody indices is visible. Serologic data were obtained for intervals of six months. At each given interval, data from 2–12 patients and 2–11 patients were available, respectively. The cut-off level is 1. Time of resection and start of chemotherapy is at month 0, respectively.

the Em^{2plus}, Em10, and Em18 assays. The Em18 index demonstrated the most marked decrease of all assays during the first follow-up interval in all patients and PNM stages in this cohort (Figure 2A).

Once the antibody levels decreased below the cut-off, they remained undetectable throughout the observation period in all assays. The decrease below the cut-off level reflected serologically the clinical regression in this patient cohort as assessed by follow-up imaging. However, the indices of the Em2^{plus}, Em10, Em18, and crude antigen ELISA did not decrease below the cut-off in 2 patients (28 and 25), 3 patients (28, 4, and 24), 5 patients (2, 13, 24, 25, and 27), and 4 patients (7, 24, 25, and 28) at the end of their follow-up periods (19 and 34 months; 19, 34, and 36 months; 30–66 months; and 19–36 months), respectively. In patients who did not seroревert in the various tests, no regrowth of parasitic tissue was detected by imaging during a follow-up period of 1.5–5.5 years. This finding also applies to the three patients in stage IV without seroreversion for three different assays each (patients 24, 25, and 28). There was no test consistently unreactive for these persons. There was no association of the PNM stage and the time to negativity in the different assays. Indices of antibodies against all antigen preparations used showed similar kinetics in this patient cohort, irrespective of the individual PNM stage.

In the three patients after non-curative resection, all antibody levels showed an initial decrease. However, an increase was demonstrated again in all assays after 21, 56, and 60 months, respectively (patients 14, 18, and 21). The crude antigen ELISA and the Em18 ELISA did not show negative results, whereas the indices of the Em2^{plus} ELISA and the Em10 ELISA decresed below the cut-off in the patient with palliative resection (patient 18) after 50 months.

In the cohort of 11 patients with unresectable lesions and stable disease who received benzimidazole therapy, antibody levels showed only a slow but steady decrease during the observation period in all assays (Figures 2B and 3B). No differences in antibody kinetics in this patient cohort in different PNM stages were observed. Antibodies against the crude antigen preparation never decreased below the cut-off. In contrast, antibodies against Em2^{plus}, Em10, and Em18 decreased below the cut-off in 4 patients (16, 6, 8, and 11), 6 patients (10, 15, 8, 16, 6, and 11), and 2 patients (8 and 11) after 8, 18, 51, and 72 months; 6, 11, 15, 21, 21, and 72 months; and 15 and 72 months, respectively. The slight decrease in antibody levels paralleled serologically the clinically stable disease in this patient cohort as assessed by follow-up imaging. The Em18 index demonstrated the most pronounced decrease of all assays during the first follow-up interval in all patients and PNM stages of this cohort (Figure 2B). There were no clinical or radiologic differences between patients who showed seroreversion in assays using recombinant or purified antigens and those who did not seroревert during the follow-up periods.

In the two patients with apparently dead lesions, antibody levels showed a steady decrese. Antibodies against the crude antigen preparation remained above the cut-off for 24 and 50 months, whereas the Em2^{plus}, Em10, and Em18-assays all

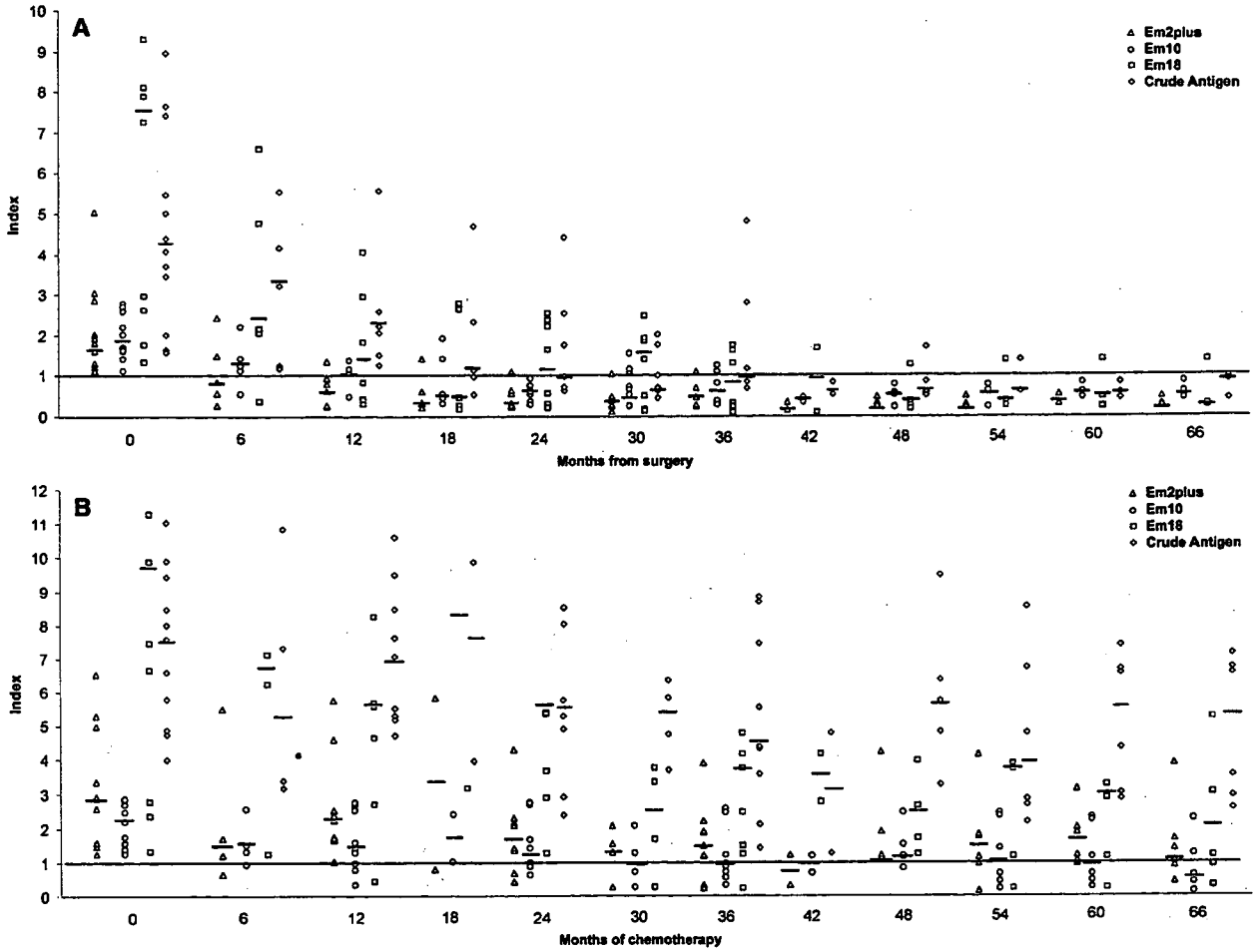

FIGURE 3. **A**, Antibody indices of all assays for 12 patients in different clinical stages after curative resection. **B**, Antibody indices of all assays for 11 patients in different clinical stages with stable disease and unresectable lesions. Serologic data were obtained for intervals of six months. At each given interval, data from 2–12 patients and 2–11 patients were available, respectively. The cut-off level is 1. Time of resection and start of chemotherapy is at month 0, respectively. Horizontal bars represent median values. High initial indices (> 14) of the Em18-assay from patients 13, 25, and 27 are not shown in **A**. High initial indices and follow-up indices (> 15) of the Em18-assay from patients 20, 22, and 26 are not shown in **B**.

showed negative results after 12 and 30 months (patients 17 and 5), respectively.

DISCUSSION

Determination of disease activity is of major importance in patients with AE. Possible recurrences should be detected timely after resection of parasitic tissue and progressive disease should be monitored closely in cases with unresectable lesions. Imaging tools enable only a retrospective evaluation of disease activity, which is reflected by growth of the metacestode. Results of real-time FDG-PET investigations are currently debated and the equipment is not readily available in many centers. No exact statement on disease activity can be given without histologic examination of excised tissue.[4] However, many areas of the parasitic lesion would have to be examined because growth of the parasitic tissue occurs focally at the periphery of the lesion(s). Surrogate parameters that have been used to monitor disease activity in AE include serum zinc concentrations,[12] total serum IgG and IgE,[12] and host alkaline phosphatase.[5] Moreover, genus-specific serologic analysis measuring IgG, IgG subclasses, and IgE directed against crude metacestode antigen preparations,[13–18] and species-specific serologic analysis measuring IgG against recombinant or purified antigens[7,8,19–21] has been used and correlation with disease activity has been demonstrated. However, patients have not been grouped according to their clinical stage.

In this study, we conducted a serologic follow-up of patients with AE grouped according to the WHO-PNM staging system with multiple tests. Four different assays were run in parallel and good agreements in the patterns of all assays were shown. The study demonstrated that the kinetics of antibody levels to *Echinococcus* were not influenced by the PNM stage. However, the height of antibody levels prior to treatment was dependent of the PNM stage. Em18 indices showed the highest, although relative weak, correlations with the PNM stages prior to treatment, a classification that is based on imaging results. As described previously, the highest antibody levels occur among inoperable cases.[20]

Hypergammaglobulinemia is a frequently encountered phenomenon in patients with AE,[22] and IgG (especially IgG4)

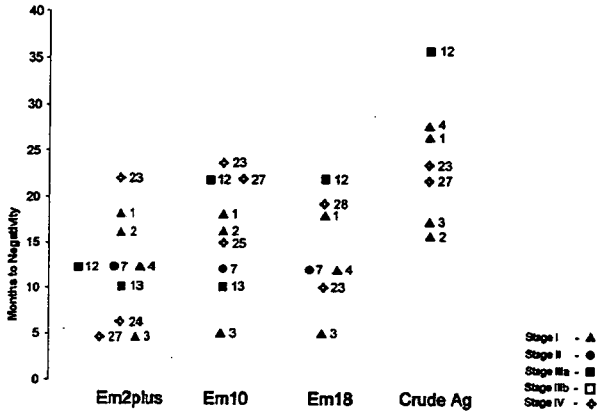

FIGURE 4. Time elapse before antibodies measured in the Em^{2plus}, Em10, Em18, and crude antigen assays fell below the cut-off in patients who underwent curative resection of echinococcal lesions. The PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, M = metastasis) stages are symbolized for each assay and patient identification numbers are shown. Levels of antibodies detected by the Em2^{plus} enzyme-linked immunosorbent assay (ELISA) decreased below the cut-off in 10 of 12 patients after 5–22 months. Antibodies detected by the Em10 ELISA decreased below the cut-off in 9 patients after 5–24 months. Antibodies detected by the Em18 ELISA decreased below the cut-off in 7 patients after 5–22 months. Antibodies detected in the crude antigen ELISA decreased below the cut-off in 8 patients after 16–60 months and remained above the cut-off longer than in any other assay. Significant differences in the time to negativity could only be measured between the Em^{2plus} assay and the crude antigen ELISA. Data of patient 13 (stage IIIa) are not shown. In this patient, the index of the crude antigen ELISA decreased below the cut-off after 60 months.

and IgE are significantly increased.[12,15–18] However, factors that determine the height of the antibody levels remain to be clarified. The activation level of the individual patient's unprotective Th2-response towards an echinococcal infection as seen in progressive cases[23] might be reflected by the amount of antibodies produced. Moreover, the duration of infection, the size and status of the lesions (i.e., metabolically active and/or growing parasitic mass), and the severity of the surrounding inflammation might all contribute to the height of the antibody levels. Parasitic tissue is in direct physical contact with host lymphoid cells[24] and an extensive accumulation of histiocytes and lymphocytes is visible in the active zone in human AE.[25] The capsule of the liver adjacent to AE lesions is often involved in the inflammatory process in form of a localized perihepatitis (frosted liver).[26] The surrounding host inflammatory zone, which is removed by curative surgery, may thus play a key role in mounting and maintaining antibody levels. Accordingly, patients with cured AE exhibit relative low humoral responses to parasitic antigen.[20]

Our study confirms previous reports that the Em18 and Em2^{plus} assays can be used for the follow-up of patients with AE. In direct comparison, antibodies against Em18 demonstrated the greatest dynamic changeability in all patients, cohorts, and PNM stages, irrespective of the individual treatment. Anti-Em10 showed similar kinetics to antibodies directed against Em^{2plus}. Thus, the Em10 assay seems suitable for serologic follow-up of patients with AE. Results comparable to those of our crude antigen ELISA have been demonstrated in the indirect hemagglutination test, which uses a hydatid fluid antigen extract in early investigations.[13] The crude antigen extract ELISA is therefore also useful for serologic follow-up of patients with AE. However, because antibodies measured by such crude antigen tests reflect much of the antigenic spectrum of the parasite, the index changes more slowly than the indices of tests that use single recombinant or affinity-purified antigens.

Our data of the patient cohort with curatively resected lesions are consistent with previous findings that levels of antibodies against Em2^{plus},[7] Em18,[8] and crude antigen preparations[13] can decrease below the cut-off. In this study, antibodies were undetectable in more patients after curative resection when measured in the Em2^{plus} ELISA than in the Em10 or Em18 ELISA, irrespective of the individual PNM stage. Many tests showed unreactive results after the same follow-up interval in some patients. None of the assays that use recombinant or purified antigens reversed significantly faster than the other after curative surgery. It is possible, however, that some tests become unreactive earlier than others, when tested in a shorter interval. Our data confirm reports of a more rapid decrease of levels of antibodies against recombinant antigens than levels of antibodies against crude antigen extracts.[27] However, the presence of viable parasitic tissue will be unlikely if the index of a test that uses a crude antigen preparation decreases below the cut-off. Some patients did not serorevert in certain assays although a recurrence during follow-up could not be demonstrated by imaging. Of this patient subgroup, most persons were in an advanced stage of AE (stage IIIa and IV, respectively). The follow-up period may not have been long enough in these patients to demonstrate either seroreversion in all assays or recurrence of disease by imaging.

In patients with recurrences, antibody levels initially decreased but increased again during the follow-up period. Similar results have been observed with the Em2^{plus} ELISA,[21] the Em18 ELISA,[8] and the Em18 Western blot.[21]

In the patient cohort with unresectable lesions, our data also mirror previous results of elevated and slowly decreasing antibody levels in the Em2,[27,28] Em2^{plus},[7] Em18,[8] and crude antigen assays.[13] In our subgroup of patients with unresectable lesions and undetectable antibodies against recombinant or purified antigens, a regression of lesions towards death of the parasite might be possible. This was shown by previous analyses for the Em2^{plus} and Em18 assays.[7,21] In our patients, the follow-up period may not have been long enough to demonstrate either seroreversion in all assays or regression of lesions by imaging.

The kinetics of antibody levels in patients who have apparently dead and fully calcified lesions were similar to the kinetics of the cohort with stable disease and no antibodies against recombinant or purified antigens.

In this study, the median follow-up period of the 28 patients examined was 5 years. A longer observation period of more patients would have been favorable in obtaining a firm basis for the correlation of serologic results and clinical condition. However, AE is a rare disease and therefore not many patients can be included in such studies.

In conclusion, serologic analysis shows a close relationship between clinical status and treatment of patients with AE. The PNM stage does not influence antibody kinetics. The Em18 index mirrors best the clinical PNM stage prior to treatment. It shows the greatest changes in all patient cohorts, clinical stages, and treatments in the early phase of medical intervention. Thus, results of the Em18 assay seem to be the easiest to interpret. The Em2^{plus} ELISA may signal successful resection

of lesions in more patients than assays using Em10 or Em18. None of the assays that use recombinant or purified antigens reverses significantly faster than the other after curative surgery. Tests employing crude antigen extracts of the parasite are the safest for detecting remaining parasitic tissue in all clinical stages. However, these tests are not useful in determining disease activity because of the long period that they need to become unreactive. Although serologic analysis is cheaper than imaging techniques, immunological test results should still be interpreted in conjunction with imaging data.

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Paragonimiasis in Cameroon: molecular identification, serodiagnosis and clinical manifestations

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Summary Paragonimiasis is a common parasitic zoonosis in Cameroon and neighbouring countries in Western Africa. Serum, sputum and faecal samples were collected in an endemic area of South West Province, Cameroon, after administration of a questionnaire to identify individuals with appropriate symptoms and histories. Microscopic examination revealed eggs in sputum from 16 people, but none in any faecal sample. These 16 were among the 25 and 26 people, respectively, positive by ELISA and by immunoblot using *Paragonimus africanus* crude antigens. Copro-DNA detection was attempted using 23 faecal samples (18 from sputum egg-negative and five from sputum egg-positive individuals). Copro-DNA was detected in four of the five sputum egg-positive individuals. These results strongly suggest that: (1) serology is much more sensitive than sputum examination for diagnosis of paragonimiasis; and (2) a copro-DNA test may

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be more sensitive than a microscopic search for eggs in faeces. Molecular sequence data from ITS2 and *cox1* genes confirmed that adult worms experimentally raised in cats were *P. africanus* and that eggs from sputum or other worm products from human faeces also belonged to this species. Based on these results, 26 of 168 persons (15.5%) were diagnosed as suffering from paragonimiasis.

1. Introduction

Paragonimiasis is a parasitic disease of humans and other mammals caused by infection with adult *Paragonimus* spp. (lung flukes). It has been estimated that 21 million people are currently infected in Southeast Asia, South America and Africa[1] and 290 million people are at risk of paragonimiasis.[2] The disease is endemic in Africa, particularly in the West and Central Equatorial region.[1,3,4] In this area, paragonimiasis and tuberculosis (TB) co-exist. The prevalence of the latter, following the spread of HIV infection, is high in Cameroon, with >300 cases per 100 000 inhabitants.[5] As the symptoms of paragonimiasis and TB are similar, potentially leading to diagnostic confusion, it is important to differentiate between them.[1]

In Cameroon, epidemiological and clinical studies on paragonimiasis based on parasitological investigations and the discovery of eggs in sputa have reported prevalences from 3.0 to 9.6%.[6–9] However, it is very difficult to detect eggs during the pre-patent period, in single-worm infections, or in cases of extrapulmonary paragonimiasis (including cerebral paragonimiasis). Furthermore, eggs are not found in every sputum sample, even in typical pulmonary paragonimiasis.[10,11] Therefore, any epidemiological survey based only on egg detection will underestimate the true prevalence.

ELISA and immunoblot have been used for serodiagnosis of paragonimiasis in Asia and Latin America.[10,12,13] In Africa, however, there have been no studies on paragonimiasis based on immunological or molecular tools.

This study was conducted to identify the *Paragonimus* species present in an endemic area in Cameroon, to compare the sensitivities of serodiagnostic and microscopic methods in diagnosis, and to evaluate a copro-DNA test for detection of eggs in faeces.

2. Materials and methods

2.1. Study sites

Four villages, Bulutu, Ebonji, Etam and Teke, were selected for this study. They are located in the Tombel Health District (50 000–100 000 inhabitants) in the rainforest zone about 40 km northwest of Kumba, Manengouba Department, South West Province of Cameroon (4°3′N, 9°3′W). The annual average temperature is 24 °C, and the relative humidity varies from 52 to 74%. Agriculture is the principal economic activity; hunting and fishing are also practiced. The study area is known to be the most endemic for paragonimiasis in Cameroon, because the population of this area habitually eats crabs.

2.2. Parasite materials and preparation of *Paragonimus* antigens

Two cats were each experimentally infected with 40 *Paragonimus* metacercariae obtained from crabs (*Sudanonautes africanus*) collected at Bulutu. Two months after inoculation, flukes were recovered from the lungs of the cats under humane conditions and used for further investigations. The frozen adult worms (0.5 g) were ground in 200 μl PBS (pH 7.2) with a Teflon-homogenizer and centrifuged (Himac CR 15, Hitachi, Tokyo, Japan) at 20 000 *g* for 10 min at 4 °C. The supernatants were used as crude antigens and stored at −20 °C. The concentration of the antigen was estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.3. Human samples

The survey was conducted in the general population in January 2004 and February 2006. The chief of each village was informed about the study and participants or parents/guardians gave informed consent for participation. People living in the area were asked whether they had experienced symptoms such as cough, haemoptysis, headache, epilepsy and chest pain, and whether they consumed raw and/or undercooked crabs. Following this, serum, sputum and faecal samples were collected from 168 people, all of who habitually ate crabs (28, 52, 55 and 33 from Bulutu, Ebonji, Etam and Teke villages, respectively). Sputum samples and faeces were examined in Cameroon for the presence of eggs to provide a diagnosis of paragonimiasis. Stool examination was performed using the formalin-ether concentration method.[14] Serum samples, a small number of faecal samples ($n = 23$) and the parasite specimens were examined in Japan. Serum samples from healthy donors from areas endemic (10 samples) and non-endemic (9 samples) for paragonimiasis were also collected. All individuals found to have eggs in the sputum were treated with praziquantel at a dose of 3 × 25 mg/kg of body weight per day for 3 consecutive days. Serum samples from confirmed *P. westermani*- ($n = 57$) and *P. miyazakii*- ($n = 7$) infected cases from Japan were also used.

For evaluation of the specificity of *P. africanus* antigens, 68 serum samples from patients with other helminthic infections were used.[15] These included *Schistosomiasis haematobium* ($n = 10$), *Schistosomiasis japonicum* ($n = 10$), *Fascioliasis* ($n = 10$), *Clonorchiasis* ($n = 19$), cysticercosis ($n = 10$) and sparganosis ($n = 9$).