models. A full heuristic search with the option of 50% majority-rule consensus was used for the tree construction, and the robustness of trees was tested by bootstrapping with 500 replicates. The ML trees were rooted with an outgroup taxon.

The Bayesian program BEAST 1.5 (Drummond and Rambaut, 2007) was run to reconstruct a taeniid phylogeny without outgroups. By using the user interface BEAUti 1.5, the program script file was prepared from the exon data set including 26 taxa. Nucleotide substitution models were selected from the output lists of Modeltest, and applied to the gene partitions of the exon data set as follows: GTR + I + G for *rpb2* and *pepck*; HKY + I for *pold*. Another script file was made by using an amino acid alignment deduced from the exon data set. The amino acid substitution model Dayhoff + G was applied to the alignment. The starting tree was randomly generated under the speciation model of Yule process. Time-calibrated branch lengths were estimated by the relaxed clock model of uncorrelated lognormal distribution (Drummond et al., 2006). In the Markov chain Monte Carlo (MCMC) options, the chain length was set to 10 million and the file log to 1000. The resultant output file containing 10,000 trees was summarized as a tree file by using TreeAnnotator 1.5 at a burn-in parameter setting of 1000. The program FigTree 1.3 (http://tree.bio.ed.ac.uk/) was used to depict phylogenetic trees.

All programs for phylogenetic inference were run at least twice to verify the consistency of the results.

3. Results

3.1. Gene structure and polymorphism

A fresh sample for the source of undegraded RNA was available only in *E. multilocularis*. Therefore, the gene structures of three nuclear markers were directly examined for this parasite. Comparisons between the sequences of cDNA and gDNA enabled identification of exon–intron boundaries. PCR target regions within each gene are displayed in Fig. 2. Each of the regions included introns as follows: two short introns in *rpb2* (a total of 162 bp in length, representing 10.7% of the whole sequence length), three short introns in *pepck* (161 bp, 10.0%) and four introns in *pold* (923 bp, 46.8%). The *pold* had longer intronic regions than the other genes. Multiple sequence alignments suggested that the target genes of the other taeniid taxa have the same gene structures with those of *E. multilocularis*. However, the lengths of introns differed between the genera *Echinococcus* and *Taenia* (data not shown). Each intron sequence was inferred to start with GT and to end with AG, except for the fourth intron of *pold* in *E. ortleppi* and *E. canadensis* genotypes, which commenced with GC. The exact structure of exon–intron boundaries and the conservation of reading frames indicated that the amplified regions of each target are not pseudogenes.

The PCR-amplified fragments of target genes were unequivocally sequenced in most samples. However, double peaks in sequence electropherograms were observed in the amplicons of *E. oligarthrus* and *T. ovis*. In particular, the three loci of *rpb2*, *pepck* and *pold* were polymorphic in *E. oligarthrus*. As the results of the cloning of the polymorphic PCR products, two alleles were detected in each locus at approximate ratio of 1:1, suggesting heterozygosity (Table 1). There were 4–6 nucleotide substitutions between the heterozygous copies, but the difference was less divergent than between species. The database accession numbers of nucleotide sequences listed in Table 1 were used for phylogenetic analyses. In cases that two alleles were found, one was randomly selected for use.

3.2. Sequence characteristics

The characteristics of nuclear gene markers were summarized by analyzing both gDNA and exon data sets (Table 3). The gDNA data set was made only for *Echinococcus* taxa. The numbers of nucleotide sites examined in the gDNA data set were 1526 for *rpb2*, 1617 for *pepck* and 1865 for *pold*. In this data set, average pairwise divergences among *Echinococcus* taxa were low for each gene (0.012–0.021). The comparison of sequence characteristics between genera was achieved by using the exon data set including both *Echinococcus* and *Taenia* taxa. The numbers of nucleotide sites examined in the exon data set were 1353 for *rpb2*, 1452 for *pepck* and 921 for *pold*. Parsimony-informative sites were more abundant in *Taenia* than in *Echinococcus*. Pairwise divergences of each gene were similar at generic level, but about 10-fold higher in *Taenia* than in *Echinococcus*.

Using the exon data set, difference statistics between synonymous and nonsynonymous substitutions (d_S–d_N) were recorded as the average of all sequence pairs within the groups of *Echinococcus* and *Taenia* (Table 3). Synonymous substitutions were predominant in all the genes, indicating negative selection against amino acid mutations. The d_S–d_N values of each gene were higher in *Taenia* than in *Echinococcus*. The deduced amino acid sequences of all the genes were conserved among taeniid taxa, and their pairwise divergence ranged as follows: 0.034–0.000 (*rpb2*); 0.088–0.000 (*pepck*); 0.140–0.000 (*pold*). According to the results of BLAST protein database search, these amino acid sequences showed high homology to the corresponding proteins, which are needed for the maintenance of basal cellular function.

3.3. Maximum likelihood (ML) phylogenetic trees

Phylogenetic relationships among *Echinococcus* taxa were inferred from the gDNA and exon data sets including 11 *Echinococcus* taxa and an outgroup (*T. mustelae*) by ML analysis. A rooted phylogram with branch lengths was inferred from the gDNA data set

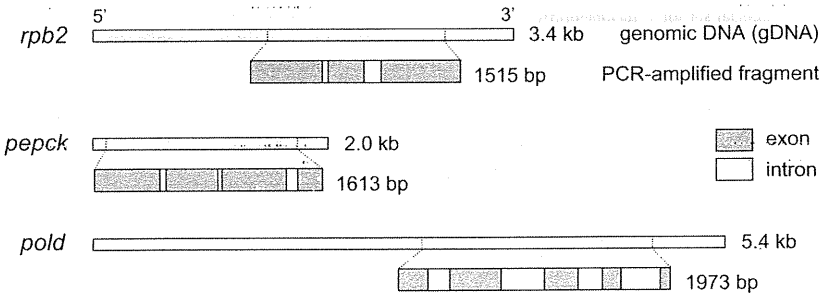

Fig. 2. PCR-amplified regions in nuclear protein-coding genes for RNA polymerase II second largest subunit (*rpb2*), phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*).

Table 3
The characteristics of nuclear gene markers used for the phylogenetic analyses of taeniid tapeworms.

Genes	N[a]	*Echinococcus*		*Taenia*		Pairwise divergence[b]		d_S–d_N[c]	
		V	*P* (%)	*V*	*P* (%)	*Echinococcus*	*Taenia*	*Echinococcus*	*Taenia*
exon[d]									
rpb2	1353	54	21 (1.55)	352	220 (16.26)	0.010	0.100	6.882*	24.075*
pepck	1452	64	16 (4.27)	397	240 (16.53)	0.011	0.106	7.262*	23.138*
pold	921	45	8 (0.87)	308	180 (19.54)	0.012	0.129	3.970*	17.108*
Total	3726	163	45 (1.21)	1057	640 (17.18)	0.011	0.109	9.841*	34.171*
gDNA[e]									
rpb2	1526	69	31 (2.03)	–	–	0.013	–		
pepck	1617	81	20 (1.24)	–	–	0.012	–		
pold	1865	167	30 (1.61)	–	–	0.021	–		
Total	5008	317	81 (1.62)	–	–	0.016	–		

[a] *N*, number of nucleotide sites examined; *V*, number of variable sites; *P* (%), number of parsimony-informative sites (percent ratio of the informative sites to the sites examined).
[b] The means of pairwise divergence values within each genus.
[c] The assessment of negative selection by codon-based Z test. The test statistics (d_S–d_N) are the average of all sequence pairs within each genus. The d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site. Significant values are indicated by an asterisk ($p < 0.01$).
[d] The intron regions of each gene were excluded from the alignment of genomic DNA (gDNA) sequences including 11 *Echinococcus* and 15 *Taenia* taxa.
[e] The alignment of gDNA sequences used for phylogeny estimation included 11 *Echinococcus* taxa and *T. mustelae* as an outgroup taxon.

(Fig. 3). The resultant phylogram clearly demonstrated that all members of *Echinococcus* spp. are closely related to one another. Three sister species pairs (*E. granulosus* s.s. and *E. felidis*; *E. multilocularis* and *E. shiquicus*; *E. oligarthrus* and *E. vogeli*) could be distinguished in the gDNA tree. In particular, *E. oligarthrus* and *E. vogeli* was a sister clade to all the other *Echinococcus* taxa. These sister species were supported with high bootstrap values, although the ILD test indicated incongruence among the gene partitions of the gDNA data set ($p = 0.006$). Another tree was constructed using the exon data set, whose gene partitions were homogeneous ($p = 0.236$). The exon tree also demonstrated that the clade of *E. oligarthrus* and *E. vogeli* is sister to all the other *Echinococcus* taxa (Fig. 3). The three sister species pairs found in the gDNA tree were also shown in the exon tree, but the node of *E. granulosus* s.s. and *E. felidis* was not supported by bootstrap resampling. In both of the gDNA and exon trees, the closely related group of *E. ortleppi* and

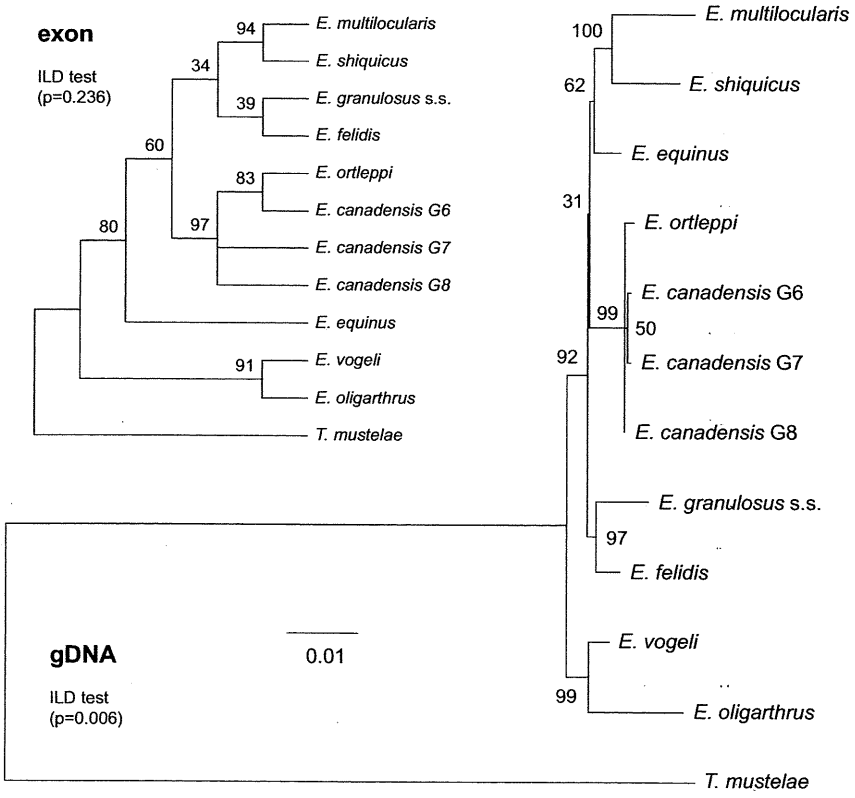

Fig. 3. Phylogenies of *Echinococcus* inferred by maximum likelihood (ML) analysis using the gDNA and exon data sets of *rpb2*, *pepck* and *pold*. Trees were rooted with *T. mustelae* as an outgroup. A phylogram with branch lengths was constructed from the gDNA data set. A cladogram was built from the exon data set. Values at each node are bootstrap proportions (%). Scale bars in the phylogram represent the estimated number of nucleotide substitutions per nucleotide site. The results of ILD test for each data set are indicated in the trees.

E. canadensis genotypes (G6–G8) was not clearly separated, indicating that the amount of nucleotide characters used was insufficient in the concatenated data sets.

In order to evaluate the potentials of *rpb2*, *pepck* and *pold* markers in resolving taeniid phylogeny, individual gene trees were made by ML method using the exon alignments of each gene including 26 taeniid taxa and an outgroup (Supplementary Fig. 1S). Each gene produced low resolution trees and their topologies were in partial conflict with one another, suggesting that the sole application of each gene marker is insufficient to reconstruct the phylogeny. However, all of the gene trees indicated that the genus *Echinococcus* is monophyletic and that the genus *Taenia* is paraphyletic.

3.4. Bayesian phylogenetic trees

Using the exon data set including 26 taeniid taxa, a Bayesian phylogenetic tree was constructed by BEAST software. The ILD test showed that the gene partitions of the data set were homogeneous ($p = 0.611$). Outgroup taxa were not included in the data set, but the BEAST determined a putative root position in the tree. According to the results of ML analyses, *Echinococcus* was treated as a monophyletic entity in the BEAST parameter settings. Moreover, the sister species pair of *E. oligarthrus* and *E. vogeli* was utilized for molecular dating. The time to the most recent common ancestor (TMRCA) of both species was assumed as a normal distribution centered at 3.0 million years ago (Ma) with a standard deviation of 0.3 Ma. This gave a central 95% range of 2.4–3.6 Ma, corresponding to the Great American Biotic Interchange (Marshall et al., 1979). The TMRCA is based on the paleozoogeographic event in which the ancestors of definitive hosts for *E. oligarthrus* and *E. vogeli* migrated from North America via Central America to South America (Rausch, 1985).

The resultant phylogenetic tree with divergence times presented a new proposal for the intra- and inter-generic relationships of taeniid parasites (Fig. 4). In the clade of *Echinococcus* taxa, the ancestral node was estimated at 5.8 Ma. The clade of *E. oligarthrus* and *E. vogeli* was sister to all the other members of *Echinococcus*. Resolution at the clade of *E. granulosus* s.s. and *E. felidis* was low, but the sister species relationship of *E. multilocularis* and *E. shiquicus* was highly supported. These topological structures were identical to those of the exon-based ML tree rooted with *T. mustelae*. The genus *Taenia* was paraphyletic in the Bayesian tree. The clade of *T. taeniaeformis* and *T. parva* belonging to the group II of Verster (1969) was placed as sister to all the other taxa including *Echinococcus*. Two highly supported major clades were formed among the *Taenia* taxa. The one was composed of human *Taenia* and related species and the other included two species of the group II (*Taenia twitchelli* and *T. martis*). The split of the two major clades was proposed to be 19.6 Ma. The remaining group II species, *T.*

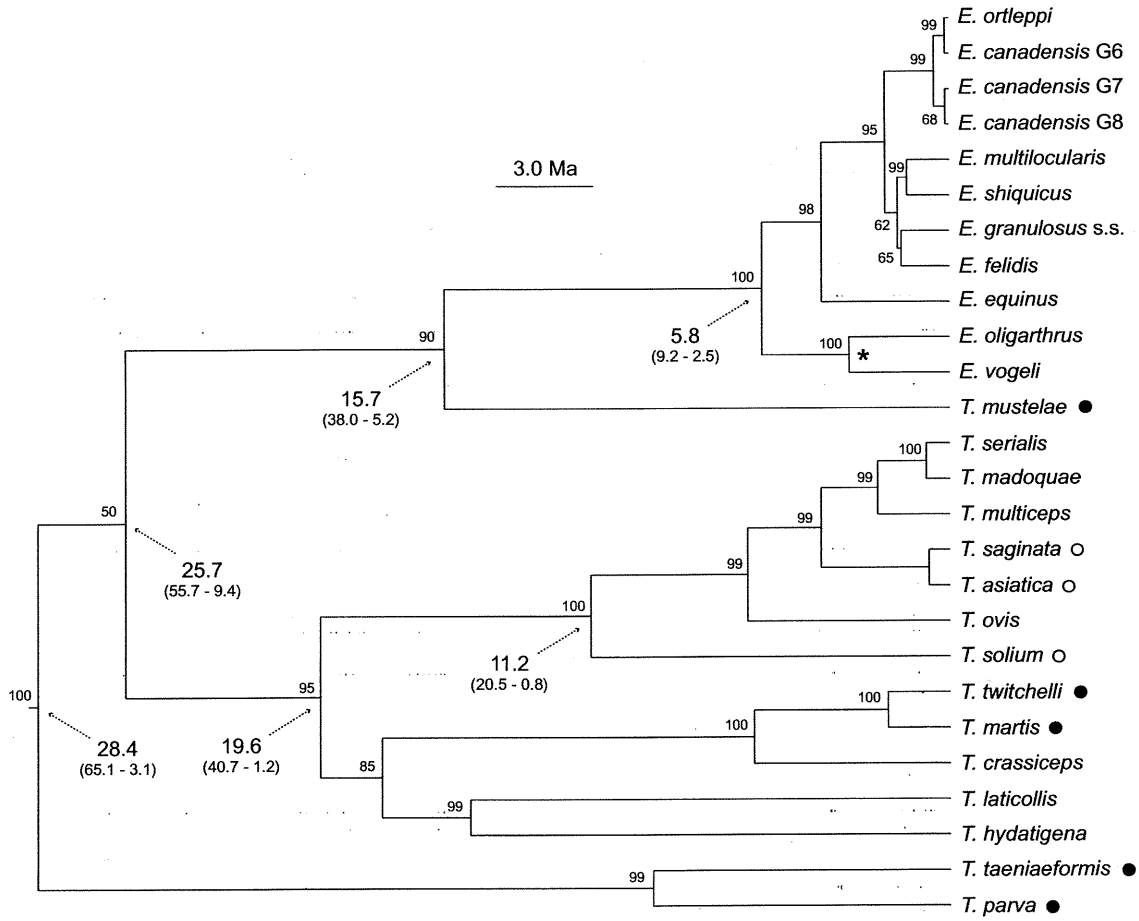

Fig. 4. A relaxed-clock chronogram of taeniid tapeworms reconstructed by Bayesian inference (BEAST) using the exon data set of *rpb2*, *pepck* and *pold*. Values at each node are posterior probabilities (%). Arrows indicate the estimated times of representative nodes (Ma, million years ago), and the heights of 95% HPD (the highest posterior density) are shown in parentheses. An asterisk denotes a node for the most recent common ancestor of Neotropical *Echinococcus* spp. used as a calibration point for time estimates. Human *Taenia* spp. are indicated by open circles, and the group II species of Verster (1969) by closed circles.

mustelae, was sister to the clade of *Echinococcus* and their ancestral node was estimated at 15.7 Ma. However, the height of 95% HPD (the highest posterior density) indicates that the estimates of the divergence time widely fluctuate in the older nodes of the tree.

Another Bayesian phylogenetic tree was constructed by using amino acid sequences deduced from the exon data set (Supplementary Fig. 2S). The tree provided low-resolution branches, particularly for the clade of *Echinococcus*. Part of the reason is probably due to the predominance of synonymous substitutions in the exon data set. A notable point in the tree is that the relatedness of *T. mustelae* with the genus *Echinococcus* was highly supported. A clade consisting of human *Taenia* and related species was kept even in the low-resolution tree.

4. Discussion

Collecting comprehensive samples of fresh organisms is difficult especially in obligate parasites infecting the internal organs of wild mammals. The phylogenetic analysis of expressed genes through the construction and sequencing of cDNA is, therefore, actually impossible for taeniid parasites. In this study we used the ethanol-preserved samples of taeniid parasites and reconstructed their phylogeny, based on the gDNA sequences of the nuclear protein-coding genes *rpb2*, *pepck* and *pold*. Putative heterozygosity at these loci was infrequent, as suggested from the autogamy of tapeworms (Lymbery, 1992). The target regions of the three gene markers contained exons and introns in various lengths. Since the alignment of gDNA sequences including *Taenia* taxa was unreliable in every gene marker, exon regions extracted from the alignment were exclusively used for phylogenetic analyses. The exon regions of each gene were assumed to be under negative selection.

Although the use of orthologous genes is needed to reconstruct nuclear phylogenetic trees, differentiation between orthologous and paralogous genes is not easy in minor taxonomic groups whose genomic sequence information is especially scarce. In this study it was difficult to demonstrate whether the three gene markers are orthologous. However, amino acid sequences deduced from the markers were highly conserved among taeniid taxa and these sequences showed high homologies to functional proteins, suggesting that the markers belong to orthologue families. Another difficulty is that incongruence between gene partitions occurred in the gDNA data set of *Echinococcus* taxa. The exon data set was, therefore, prepared by removing intron regions to obtain homogeneity between gene partitions. Phylogenetic trees inferred from both the data sets provided relatively similar results for the interspecific relationships of *Echinococcus*. A slight discrepancy in their topology indicates that the gene markers used do not contain sufficient phylogenetic information to fully resolve the phylogeny of *Echinococcus*.

The taxonomy of *Echinococcus* has remained controversial over several decades because of the paucity of adult morphological characters and the great plasticity of cystic larvae developed in intermediate and aberrant hosts (Thompson and McManus, 2002). Host specificity has been regarded as a desirable property for the delineation of species and strains (Thompson et al., 1995). As shown in Fig. 1, a common phylogenetic tree of *Echinococcus* summarized from mtDNA sequence data (Nakao et al., 2007; Hüttner et al., 2008) indicates that: (1) the Neotropical species *E. oligarthrus* is sister to all other members of *Echinococcus;* (2) sister species relationships are evident between *E. multilocularis* and *E. shiquicus*, *E. granulosus* s.s. and *E. felidis*, and *E. ortleppi* and *E. canadensis*; (3) *E. equinus* forms a clade with *E. shiquicus*, *E. multilocularis*, *E. ortleppi* and *E. canadensis* (4) genotypes of *E. canadensis* are closely related to each other. The nuclear phylogenies of *Echinococcus* presented in this report strongly support the sister species relationships found in the mitochondrial phylogeny. Furthermore, the nuclear phylogenies indicate that *E. oligarthrus* is sister species to *E. vogeli*, and that both species are sister to all other *Echinococcus* taxa.

In contrast, Saarma et al. (2009) reported another nuclear phylogenetic tree made by Bayesian inference. Data sets used for this study included the protein-coding genes of ezrin–radixin–moesin-like protein (*elp*), elongation factor 1 alpha (*ef1a*), transforming growth factor beta receptor kinase (*tgf*), thioredoxin peroxidase (*th*) and calreticulin (*cal*); however, the sequence data of *tgf*, *th* and *cal* were lacking in several taxa. The resulting phylogeny revealed that *E. multilocularis* is sister to all other members of *Echinococcus* (Fig. 1). Nevertheless, the present nuclear and previous mitochondrial trees demonstrated a sister species relationship between *E. multilocularis* and *E. shiquicus*. Both species are closely related to each other, even in the properties of host specificity and morphology (Xiao et al., 2005). Another important difference is that this study found the cryptic species complex of *E. granulosus* s.s., *E. equinus*, *E. felidis*, *E. ortleppi* and *E. canadensis* as a paraphyletic entity, whereas in Saarma et al. (2009) they were monophyletic. In spite of these critical discrepancies, phylogenetic trees of both studies have high support values for most of the important nodes. A possible explanation of the discrepancies is that the tree topology of Saarma et al. (2009) was inferred in the absence of outgroup taxa. Further analyses with even more nuclear gene markers are required to unequivocally resolve the phylogeny of *Echinococcus*.

In this study the Bayesian phylogeny of taeniid parasites indicates that most of *Taenia* taxa are divided into two major clades. The branching pattern of a clade including human *Taenia* spp. supports the previous finding that *T. solium* is distantly related to *T. saginata* and *T. asiatica* (Hoberg et al., 2000). Another important point of this clade is that *Taenia multiceps*, *Taenia serialis* and *Taenia madoquae* are more closely related to *T. saginata* and *T. asiatica* than to *T. solium*. This topology is supported by other phylogenetic analyses using mtDNA (de Queiroz and Alkire, 1998; Gasser et al., 1999; Zhang et al., 2007; Lavikainen et al., 2008; Jia et al., 2010). However, numerical taxonomic analyses using morphological characters (Hoberg et al., 2000; Hoberg, 2006) suggest that *T. multiceps* and *T. serialis* are distantly related to the three species of human *Taenia*. As mentioned in the "Out of Africa" hypothesis of human *Taenia* (Hoberg et al., 2001), *T. saginata* and *T. asiatica* share a common ancestor with the lion tapeworm *Taenia simbae*, while *T. solium* is closely related to the hyena tapeworms *Taenia hyaenae* and *Taenia crocutae*. The genetic makeups of these African wildlife parasites are required to clarify the discrepancy between the molecular and morphological phylogenies. The evolutionary history of human *Taenia* may be improved by adding the molecular data of the African species.

As concerns the intergeneric relationship of taeniid parasites, the present Bayesian tree represents an important finding that the genus *Taenia* is paraphyletic. The group II species of Verster (1969) such as *T. taeniaeformis*, *T. parva* and *T. mustelae* played a critical role in resolving the paraphyly. The clade of *T. taeniaeformis* and *T. parva* is sister to all the other *Taenia* and *Echinococcus* taxa. Moreover, *T. mustelae* is sister to the genus *Echinococcus*. However, the remaining taxa of the group II, *T. twitchelli* and *T. martis*, formed a different clade together with *Taenia crassiceps*, suggesting that the arrangement of genital duct used for the grouping of *Taenia* (Verster, 1969) is not a synapomorphy. The duct variation seems to occur several times independently in taeniid evolution. The placement of *T. crassiceps* as a sister of *T. martis* and *T. twitchelli* is identical to that in a mtDNA phylogeny (Lavikainen et al., 2008). The close relationships among the three species are also supported by numerical taxonomic analyses using morphological characters (Hoberg et al., 2000; Hoberg, 2006).

In previous taxonomic treatments, *T. taeniaeformis* and *T. parva* were placed in the genus *Hydatigera* Lamarck 1819, and the first one was ranked as the type species of the genus (Abuladze, 1964). Also *Taenia laticollis* had been classified into *Hydatigera*, but our data indicate that it is distantly related to *T. taeniaeformis* and *T. parva*. According to Abuladze (1964), *T. parva* and *T. laticollis* might not be included in *Hydatigera* due to morphological differences in the neck region of the strobila. The present phylogenetic study suggests a possibility that *T. taeniaeformis* and *T. parva* could be placed in a distinct genus. In terms of priority, it should be called *Hydatigera*. However, numerical taxonomies based on morphological characters do not recognize the validity of *Hydatigera* (Hoberg et al., 2000; Hoberg, 2006). In addition, the position of *T. taeniaeformis* in several mtDNA-based phylogenies does not support *Hydatigera* either (Okamoto et al., 1995; de Queiroz and Alkire, 1998; von Nickisch-Rosenegk et al., 1999b), or its status has remained unsolved (Zhang et al., 2007; Lavikainen et al., 2008).

In morphology-based phylogenies of the genus *Taenia* (Hoberg et al., 2000; Hoberg, 2006), *T. mustelae* is the most distant from the other species. Furthermore, a mtDNA-based phylogeny suggests that *T. mustelae* is sister to the genus *Echinococcus* (Lavikainen et al., 2008). This genetic relatedness is strongly supported by our nuclear phylogeny. Although further taxon sampling is needed to evaluate the ancestral lineages of *Echinococcus*, the present data suggest that a new genus should be created at least for *T. mustelae*. It is difficult to determine clear morphological synapomorphies between *T. mustelae* and the genus *Echinococcus*. The tapeworms of *Echinococcus* spp. are characterized by a tiny strobila (up to 1.2 cm in length) consisting only of a few segments (Abuladze, 1964; D'Alessandro and Rausch, 2008). Although *T. mustelae* is one of the smallest species of *Taenia*, it consists of dozens of segments and is gigantic (up to 15 cm) compared to *Echinococcus* (Abuladze, 1964). A possible synapomorphy shared by *T. mustelae* and *Echinococcus* spp. could be the small size of the scolex and related structures. The diameter of the scolex, rostellum and suckers, as well as lengths of the rostellar hooks fall into the same range in *T. mustelae* and *Echinococcus* spp. (Abuladze, 1964; Loos-Frank, 2000; Ortlepp, 1934; Rausch and Berstein, 1972; Rausch and Nelson, 1963; Schantz and Colli, 1973; Verster, 1969; Xiao et al., 2005). These structures are in general bigger in other *Taenia* spp. However, the small scolex of *Echinococcus* could be just a consequence of the small overall size of the strobila. The larval stages of *Echinococcus* (hydatids) are absolutely proliferative, but *T. mustelae* can form both uniscolex cysticerci and multiscolex coenuri (Locker, 1955; Freeman, 1956). The potential of larval stages to multiply should be considered as a plesiomorphy since proliferative larvae occur in some other species of *Taenia* (Loos-Frank, 2000).

The geographic ranges of parasites infecting wildlife animals are limited by host distributional patterns, but those infecting domestic animals often show cosmopolitan distribution because of anthropogenic movements of host animals. The sister species pair of *E. oligarthrus* and *E. vogeli* is distributed only in the tropical forests of Central and South America, since carnivores and rodents indigenous to the Neotropical ecozone serve as their specific hosts (D'Alessandro and Rausch, 2008). The endemism of both the species played a key role for the present chronological study on taeniid parasites. The Great American Biotic Interchange around 3.0 Ma was used as a critical point for the time calibration of Bayesian relaxed-clock dating. Before the formation of the Panamanian land bridge, no mammals of the order Carnivora prevailed in South America (Simpson, 1980). It therefore seems most likely that the land bridge-mediated disturbance caused the invasion of carnivores to Central and South America together with a common ancestor of *E. oligarthrus* and *E. vogeli*. Setting the TMRCA of both the parasites to 3.0 Ma yielded a molecular chronogram spanning *Echinococcus* and *Taenia*. This is the first case in which the divergence times of tapeworms have been estimated by a molecular phylogenetic approach using a geological event as a yardstick. The chronogram based on nuclear protein-coding genes suggests that a clade of *Taenia* including human-pathogenic species had diversified primarily in the late Miocene (11.2 Ma), whereas *Echinococcus* had begun to diversify later, in the end of the Miocene (5.8 Ma). Close genetic relationships among the members of *Echinococcus* imply that the genus is a young group in which speciation and global radiation had occurred rapidly. The members of the family Taeniidae are obligate parasites of mammals, indicating that taeniid evolution totally depends on the adaptive radiation of mammals and on the structure of the predator-prey relationships. The evolution of the order Carnivora is especially important in considering the dispersal of the parasites. The adaptive radiation of mammals started at the beginning of the Cenozoic era (65 Ma), and modern canines and felines appeared from Miocene epoch (23–5 Ma) (Wayne et al., 1997; Johnson et al., 2006). The time estimates of taeniid evolution appear to be acceptable when compared with the time range of mammalian evolution, but other settings of TMRCA are needed for further calibration, particularly in *Taenia* taxa.

The putative single copy genes shown in this study may be available as population genetic markers to examine the following problematic issues on *Echinococcus* and *Taenia* taxonomy. The classification of *E. canadensis* genotypes is always a debatable issue (Thompson et al., 2006; Lavikainen et al., 2006; Nakao et al., 2007; Moks et al., 2008; Thompson, 2008; Saarma et al., 2009). Mitochondrial and nuclear phylogenies in these studies raised a basic question whether the genotypes G6 and G7 (pig and camel strains) are different from G8 and G10 (cervid strains) at species level. The cervid strains are ecologically and geographically segregated by colonizing sylvan wildlife (Rausch, 2003), suggesting the difference to be at subspecies level. In this study, the gDNA sequences of *rpb2* were identical among the genotypes G6, G7 and G8. However, nucleotide substitution sites to differentiate these genotypes were found in *pepck* and *pold*. The population genetic structures of *E. canadensis* genotypes examined by these and additional nuclear gene markers may provide a basis for judging their species status. Another problematic issue is the gene introgression of taeniid parasites. Possible cases of gene introgression have been reported in *Echinococcus* (Badaraco et al., 2008) and *Taenia* (Okamoto et al., 2010), suggesting that reproductive isolation is incomplete among closely related species. The nuclear gene markers will be also helpful in analyzing the heterozygosity of individual parasites collected from areas where related species are coexisting.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.07.022.

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Echinococcus multilocularis: Identification and functional characterization of cathepsin B-like peptidases from metacestode ☆

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ABSTRACT

Cysteine peptidases have potent activities in the pathogenesis of various parasitic infections, and are considered as targets for chemotherapy and antigens for vaccine. In this study, two cathepsin B-like cysteine peptidases (EmCBP1 and EmCBP2) from *Echinococcus multilocularis* metacestodes were identified and characterized. Immunoblot analyses demonstrated that EmCBP1 and EmCBP2 were present in excretory/secretory products and extracts of *E. multilocularis* metacestodes. By immunohistochemistry, EmCBP1 and EmCBP2 were shown to localize to the germinal layer, the brood capsule and the protoscolex. Recombinant EmCBP1 and EmCBP2 expressed in *Pichia pastoris*, at optimum pH 5.5, exhibited substrate preferences for Z-Phe-Arg-MCA, Z-Val-Val-Arg-MCA, and Z-Leu-Arg-MCA, and low levels of hydrolysis of Z-Arg-Arg-MCA. Furthermore, recombinant enzymes degraded IgG, albumin, type I and IV collagens, and fibronectin. These results suggested that EmCBP1 and EmCBP2 may play key roles in protein digestion for parasites' nutrition and in parasite–host interactions.

1. Introduction

Alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus multilocularis*, is a serious parasitic disease of humans in countries of the higher latitudes of Northern Hemisphere. In the previous decade, a lot of new data have been published on prevalence of *E. multilocularis* in final and intermediate hosts in areas where it had previously not been recorded (Eckert et al., 2000; Ito et al., 2010). Humans are accidentally infected with *E. multilocularis* by ingestion of eggs excreted with the feces of carnivores harboring adult tapeworm of this species. It is thought that humans become exposed to *E. multilocularis* by handling of infected definitive hosts, or by ingestion of food and water contaminated with eggs. Oncospheres hatched from eggs in the small intestine of humans migrate via the portal system into various organs, mainly liver and differentiate and develop into the metacestode stage. The metacestodes propagate asexually like a tumor leading to organ dysfunction. Since clinical symptoms usually do not become evident until 10 or more years after initial parasite infection, early diagnosis and treatment especially during asymptomatic period are important for reduction of morbidity and mortality (McManus et al., 2003). About one third of patients have cholestatic jaundice and about one third of patients have epigastric pain. In the remaining patients, *E. multilocularis* infections are incidentally detected during medical examination for symptoms such as fatigue, weight loss, hepatomegaly (Pawlowski et al., 2001). In addition to surgical removal of alveolar hydatid cyst, treatment with antiparasitic agents, benzimidazole derivatives, is the most important for AE therapy. However, these drugs have parasitostatic activity rather than parasitocidal activity, and side-effects such as liver damage are often observed with long-term administration (Kern, 2006). Thus, it is urgent to develop novel reliable chemotherapeutic agents.

Cysteine peptidases belonging to clan CA family C1 (Rawlings et al., 2004; http://merops.sanger.ac.uk/) besides their housekeeping functions such as protein turnover in parasite cells are involved in evasion from host immune responses, essential nutrient uptake, and tissue penetration, by degrading host proteins, including immunoglobulin, complement components, kininogen, haemoglobin, albumin, and extracellular matrix proteins (reviewed by Sajid and McKerrow 2002; Dalton et al., 2003; Caffrey et al., 2004; Rosenthal, 2004; McKerrow et al., 2006; Robinson et al., 2008; Smooker et al., 2010). Furthermore, some cysteine peptidases have activities to stimulate human eosinophils to induce degranulation (Shin et al., 2005), deplete CD4 positive human lymphocytes *in vitro* (Molinari et al., 2000), induce apoptosis in human CD4 positive cells (Tato et al., 2004), by interacting with host cells via unknown mechanisms. Therefore, cysteine peptidases of parasites are considered as important targets for chemotherapy and/or

☆ *Note*: The nucleotide sequence data reported in this study are available in the GenBank, EMBL, and DDBJ databases under accession numbers AB586072 (EmCBP1) and AB586073 (EmCBP2).

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immunoprophylaxis (Dalton et al., 2003; Barr et al., 2005; Abdulla et al., 2007; Alcala-Canto et al., 2007).

E. multilocularis metacestodes survive for many years in human host, which leads us to consider peptidases as important parasite components to evade from host immune responses and to uptake nutrient. However, precise characterization of *E. multilocularis* peptidases essential to develop enzyme inhibitors has been hampered by the difficulty of obtaining pure parasite materials, since parasite materials obtained from laboratory animals are contaminated with numerous host cells resulting from that *E. multilocularis* infiltrate and proliferate by exogenous budding of the germinative cells in host tissue (Thompson, 1995). Recently, we have succeeded in cloning of cathepsin L-like peptidase genes (EmCLP1 and EmCLP2) from *E. multilocularis* metacestodes, which enabled us to prepare a large amount of parasite enzymes for detailed characterizations (Sako et al., 2007). Activities of recombinant EmCLP1 and EmCLP2 to degrade human IgG, bovine albumin, type I and type IV collagen and fibronectin have been demonstrated, which suggests their important roles in parasite growth and survival in the host. In the present study, we have identified two genes encoding cathepsin B-like cysteine peptidases from *E. multilocularis* metacestodes. Immunoblot analyses with monoclonal antibody detected both enzymes in crude metacestode extract and ES products, and immunohistochemical studies revealed that both enzymes are expressed in the germinal layer, the brood capsule, and the protoscolex. Moreover, enzymatic activities against synthetic peptide substrates and macromolecule proteins were also characterized by using recombinant active enzymes expressed in *Pichia pastoris*.

2. Materials and methods

2.1. Animals

Animal procedures and management protocols in this study were approved by the Ethics Committee of Asahikawa Medical University, Asahikawa, Japan.

2.2. Preparation of parasite material

E. multilocularis (Furano isolate, Hokkaido, Japan) metacestode tissue was obtained from non-obese diabetic severe combined immunodeficiency (NOD/Shi-*scid*) mice infected by intraperitoneal passage of metacestodes (Nakaya et al., 2006). Microvesicle and protoscolex suspensions were prepared by pressing metacestode tissue through a 300 μm metal mesh with PBS. The microvesicles and protoscoleces were washed five to seven times with PBS, and then used for preparation of metacestode crude lysate and excretory/secretory (ES) products. Because NOD/Shi-*scid* mice had little inflammatory response, isolation of microvesicles and protoscoleces with less host components, which are commonly found in those from immunocompetent mice, could be done efficiently.

To prepare metacestode crude lysate, microvesicles and protoscoleces were homogenized with three times volume of lysis buffer consisting of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1.0% 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonic acid (CHAPS) in the presence of peptidase inhibitors (protease inhibitor cocktail for mammalian tissues, Sigma–Aldrich). After one freeze–thaw cycle and centrifugation at 10,000*g* for 30 min at 4 °C, the supernatant was recovered and kept at −80 °C until use.

To obtain ES products, microvesicles and protoscoleces were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C for 12 h. Few dead microvesicles and protoscoleces were found under a microscopic examination at the end of cultivation, which indicated that the contamination of intracellular proteins released into the medium supernatant as a result of parasite death had been almost completely avoided. The medium supernatant containing ES products was carefully collected and was passed through a disposable chromatography column (Econo-Pac column, Bio-Rad) with a porous bed support (a 30 μm pore size) to remove minor microvesicle and protoscolex contaminants. After filtration through 0.45 μm filter membrane (Millipore), the medium supernatant was concentrated by using an Amicon Ultra-15 Centrifugal Filter Unit with a cutoff size of 5 kDa (Millipore) and kept at −80 °C until use.

2.3. Cloning of EmCBP1 and EmCBP2 genes

Total RNA was isolated from freshly prepared *E. multilocularis* metacestodes using Trizol reagent (Gibco BRL) according to the manufacture's instruction. After purification of Poly(A)$^+$ RNA by using oligo(dT)-latex beads (TaKaRa), cDNA available in 5′ and 3′ rapid amplification of cDNA end (RACE) method was synthesized from 1 μg of purified poly(A) $^+$ RNA by using the GeneRacer Kit (Invitrogen).

3′ RACE were performed with degenerated forward primers designed from the consensus sequences flanking the active site residues of eukaryotic cysteine peptidases and 3′ RACE primer.

The forward primer (5′-CAGGGTCAGTGYGGNTCNTGYTGG-3′) and GeneRacer 3′ primer (5′-GCTGTCAACGATACGCTACGTAACG-3′) were used in the first round PCR, and the forward primer (5′-CAGTGCGGTTCNTGYTGGGCNTTY-3′) and GeneRacer 3′ Nested primer (5′-CGCTACGTAACGGCATGACAGTG-3′) were used in the nested PCR. PCR reactions were performed in a 50 μl of reaction mixture containing 1 × *Ex Taq* Buffer, 2.0 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP, 5 ng of cDNA and 0.5 units of *EX Taq* DNA polymerase (TaKaRa) and cycling conditions were 30 s at 94 °C (first cycle: 2 min at 94 °C), 30 s at 50 °C and 30 s at 72 °C for 30 cycles. The PCR products were separated in a 1.0% agarose gel, the DNA fragments were recovered and cloned into pGEM T-vector (Promega), and plasmid clones were sequenced. To obtain sequence upstream of EmCBP1 and EmCBP2 genes, 5′ RACE was performed using gene-specific primer (5′-CGTACCATCACTGCTC TCCCGCTTACTGTC-3′ for EmCBP1gene, 5′-TGCAACCAAAGCCACAG AATAAGCC-3′ for EmCBP2 gene) and GeneRacer 5′ primer (5′-CG ACTGGAGCACGAGGACACTGA-3′) with annealing temperature of 60 °C. Finally, full-length cDNAs of the EmCBP1 and EmCBP2 genes were cloned by PCR using a high-fidelity DNA polymerase, Phusion DNA polymerase (Finnzymes), and primers directed to both the UTR ends.

2.4. Expression of the mature region of EmCBP1 and EmCBP2 in Escherichia coli and purification

The mature enzyme region of EmCBP1 or EmCBP2 was amplified by PCR with primer sets containing a restriction enzyme (underlined) recognition sequence added to 5′ end to facilitate cloning of the PCR products. The primers used were: 5′-GGGAATTC CTGCCGGCATCTTTTGATCCC-3′ (mCBP1/F), 5′-GGGTCGACTAGTT TTGTGGGATACCTGC-3′ (CBP1/R), 5′-GGGAATTCCTTCCCTCAGAAT TTGACGCA-3′ (mCBP2/F), 5′-GGGTCGACTCACTTCCTTATTTTTGGA ATACC-3′ (CBP2/R). The PCR reactions were performed with cDNA clones as templates. The PCR products were digested with *Eco*RI and *Sal*I, cloned into bacterial expression vector pET-30a(+) (Novagen) for producing a fusion protein with His tag. The cloned plasmids were transfected into *E. coli* BL21(DE3)pLysS strain. Expression of recombinant proteins was induced by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) to the culture. Recombinant proteins were purified using Ni-NTA beads (Qiagen) under denaturation conditions. Protein concentration was determined by BCA protein assay kit (Pierce).

2.5. Production of monoclonal antibodies

Female BALB/c mice were immunized by intraperitoneally (i.p.) injection of 50 μg of *E. coli*-expressed recombinant EmCBP1 (eEmCBP1) or EmCBP2 (eEmCBP2) emulsified in Freund's complete adjuvant. Three weeks later the procedure was repeated but with Freund's incomplete adjuvant. Three days before the fusion, the mice were i.p. boosted with 50 μg antigens in PBS. Spleen cells of mice were fused with NS-1 myeloma cells. The antibody-secreting hybridomas were screened by ELISA with eEmCBP1 or eEmCBP2. Hybridomas selected were cloned by limit dilution at least twice.

2.6. SDS–PAGE and immunoblot analysis

Proteins were treated with a SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2.0% SDS, 50 mM dithiothreitol and 10.0% glycerol) at 100 °C for 5 min and separated in a 7.5% or 12.5% polyacrylamide gel. For immunoblot analysis, the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane sheet (Millipore). The sheet was blocked with blocking solution (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1.0% casein, 0.1% Tween 20) and probed with monoclonal antibody followed by alkaline phosphatase-conjugated anti-mouse IgG antibody (Novagen). Nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate was used for color development.

2.7. Immunohistochemistry

Parasite tissues and livers from infected-NOD/Shi-*scid* mice were washed once with PBS and fixed with 2.0% paraformaldehyde in PBS overnight at 4 °C and then embedded in paraffin. Sections of 5 μm were produced and were transferred to slides. After antigen retrieval using HistoVT One (Nacalai Tesque), sections were treated with peroxidase blocking solution (0.3% H_2O_2 in methanol) for 30 min. Then, sections were washed with PBS, blocked using blocking solution for 1 h and incubated overnight at 4 °C with monoclonal antibody. After three washing with PBS, the sections were incubated with peroxidase-conjugated anti-mouse IgG antibody (ImmPRESS REAGENT, Vector laboratories) for 30 min at room temperature. After four washing with PBS, the sections were incubated with 3-amino-9-ethylcarbazole. All sections were counterstained with hematoxylin.

2.8. Expression of EmCBP1 and EmCBP2 in P. pastoris

The pro-mature coding region of EmCBP1 or EmCBP2 was amplified by PCR with primer sets containing a restriction enzyme (underlined) recognition sequence. The primers used were: 5′-CGGAATTCA GTACTGTGACCAGCGCAATTGG-3′ (proCLP1/F), 5′-ATGCGGCCGCTA GTTTTGTGGGATACCTGC-3′ (PIC CLP1/R), 5′-CGGAATTCAGAAAACC TCATCAGAGCGAC-3′ (proCLP2/F), 5′-ATGCGGCCGCTCACTTCCTTA TTTTTGGAATACC-3′ (PIC CBP2/R). The PCR reactions were carried out as mentioned above. The PCR products were digested with *Eco*RI and *Not*I and cloned into yeast expression vector pPICZα A (Invitrogen), and subsequently linearized with *Pme*I and electroporated into *P. pastoris* KM71 host cells. Yeast transformants were cultured in 500 ml of buffered-glycerol complex medium (1.0% yeast extract, 2.0% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1.0% glycerol, and 100 mM potassium phosphate, pH 6.0) at 28 °C for 2 days and collected by centrifugation at 1000*g* for 5 min, and protein expressions were induced by resuspending the cells in 100 ml of buffered-methanol minimal medium (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1.0% methanol, and 100 mM potassium phosphate, pH 6.0) at 28 °C for 3 days. Due to the presence of an α-factor leader peptide sequence, recombinant proteins were secreted into expression medium. The culture medium containing recombinant EmCBP1 (yEmCBP1) or EmCBP2 (yEmCBP2) was collected, concentrated using an Amicon stirred cell with a YM-10 membrane and dialyzed against 50 mM sodium acetate buffer (pH 4.5) containing 2.5 mM EDTA. For purification of active form of yEmCBP1, dialysate was directly loaded on a HisTrap SP XL cation-exchange column pre-equilibrated with 50 mM sodium acetate buffer (pH 4.5) containing 2.5 mM EDTA after activation at 37 °C for 1 h in the presence of 10 mM L-cysteine and proteins were eluted by use of a linear salt gradient (0–1.0 M NaCl). For purification of active form of yEmCBP2, the conversion of pro-form into active enzyme was accomplished by treatment with pepsin. After addition of porcine pepsin (Sigma–Aldrich) at a final concentration of 0.5 mg/ml, the activation mixture was incubated at 37 °C for 4 h. The activated yEmCBP2 was purified as mentioned above. Recombinant proteins were treated with peptide:*N*-glycosidase F (PNGase F, New England Biolabs) under denaturing conditions to remove any N-linked oligosaccharides to determine whether recombinant proteins were glycosylated.

2.9. Irreversible active site-labelling of yEmCBP1 and yEmCBP2

A biotinylated dipeptidyl fluoromethylketone (Biotin-Phe-Ala-FMK, MP Biomedicals), a cysteine peptidase inhibitor, was used for active-site labelling. Briefly, the purified enzyme was incubated for 30 min at room temperature with 10 μM biotin-Phe-Ala-FMK in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mM EDTA, 0.1% CHAPS and 10 mM L-cystine. Labelled proteins were detected with alkaline phosphatase-conjugated streptavidin (Novagen).

2.10. Substrate specificity and kinetic measurements of yEmCBP1 and yEmCBP2

Peptidase activity was characterized by using peptidyl-4-methylcoumarin-7-amide (MCA) as substrates. The standard assay volume was 200 μl, using 100 mM sodium acetate buffer (pH 5.5) containing 0.1% CHAPS, 2.5 mM EDTA and 10 mM L-cysteine. Substrates were added to a final concentration of 2 μM, or other concentration as required. Assays were performed at room temperature. The amount of 7-amino-4-methylcoumarin (AMC) released was measured by the fluorometer (VersaFluor Fluorometer, Bio-Rad) at an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

For determination of the optimum pH of recombinant enzyme activity, 100 mM citrate–phosphate buffer (pH 3.0–8.0) containing 250 mM NaCl, 0.1% CHAPS, 2.5 mM EDTA and 10 mM L-cystine, were used. Substrate specificities were investigated using benzyloxycarbonyl (Z)-Phe-Arg-MCA, Z-Arg-Arg-MCA, Z-Leu-Arg-MCA, Z-Gly-Pro-Arg-MCA, and Z-Val-Val-Arg-MCA at a concentration of 2 μM. The values of K_m and V_{max} for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were determined by a nonlinear regression analysis. The molar concentration of active recombinant enzymes was determined by active-site titration using the Z-Phe-Arg-MCA and cysteine peptidase inhibitor *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) as described by Barrett and Kirschke (1981). All peptidyl-MCA substrates used were purchased from Peptide Institute, Japan.

2.11. Inactivation kinetics of yEmCTB1 and yEmCTB2

Inactivation of recombinant enzyme was performed in 100 mM citrate–phosphate buffer (pH 6.5, 7.0, and 7.5) containing 250 mM NaCl, 0.1% CHAPS, 2.5 mM EDTA, and 10 mM L-cystine in the presence of 50 μM Z-Phe-Arg-MCA. Progress of the reaction was monitored continuously by the fluorescence of the released products for 30 min. All progress curves obtained were exponential, and could be best fitted to the following first-order relationship (Eq. 1):

$$P = P_{\infty}(1 - e^{k_{obs}t})$$

where P and P_{∞} are the product concentration at a given or infinite time, respectively, and k_{obs} is the observed first-order inactivation rate constant. And half-lives were calculated as $t_{1/2} = \ln 2/k_{obs}$.

2.12. Protein substrate digestion

Human IgG, human serum albumin (HSA), soluble calf skin type I collagen, human placenta type IV collagen, and bovine plasma fibronectin were used as protein substrates. All protein substrates used were purchased from Sigma–Aldrich, Japan. Active recombinant enzyme (0.2 μM) was incubated with 0.2 mg/ml of each protein substrate in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mM EDTA and 10 mM L-cysteine at 37 °C for 4 h. The digestion reaction was stopped by the adding of SDS–PAGE sample buffer. Protein substrate digests were subjected to SDS–PAGE and degradation products were visualized by Coomassie Blue staining.

3. Results

3.1. Primary structure of EmCBP1 and EmCBP2

Following PCR with degenerate primers and sequencing analysis of 48 PCR product clones, a total of four different partial genes encoding cysteine peptidases were obtained from *E. multilocularis* metacestode cDNA. Database search of the deduced amino acid sequences of individual clones revealed that two clones are identical to EmCLP1 and EmCL2 (Sako et al., 2007), respectively, and other two clones show high homology to cathepsin B-like peptidases. Rapid amplification of cDNA ends (RACE) was performed to obtain the full-length of two novel cysteine peptidase cDNAs, termed EmCBP1 and EmCBP2, respectively.

As shown in Fig. 1, EmCBP1 consists of a 18-residue putative signal sequence predicted by the method of Nielsen et al. (1997), a 77-residue propeptide and the 256-residue mature enzyme. EmCBP2 consists of a 16-residue putative signal sequence, a 67-residue propeptide and the 255-residue mature enzyme. Comparison of EmCBP1 with EmCBP2 revealed an amino acid identity of 60.5% (65.6% for the mature region only). The catalytic triad residues are conserved with other crucial residues shaping an oxyanion hole (Menard et al., 1991), and the occluding loop region (Musil et al., 1991) responsible for peptidyldipeptidase activity, a unique feature of cathepsin B, is also present in the mature enzyme. The predicted molecular masses of the mature EmCBP1 and EmCBP2 are 28,241 and 28,140 Da, respectively. In mature region, EmCBP1 has one putative N-linked glycosylation site at position 116.

3.2. Detection of EmCBP1 and EmCBP2 in E. multilocularis

Immunoblot analyses of *E. multilocularis* metacestode crude lysate (Fig. 2, lanes 1) and ES products (lanes 2) were performed. Anti-EmCBP1 monoclonal antibody recognized proteins of 24.5

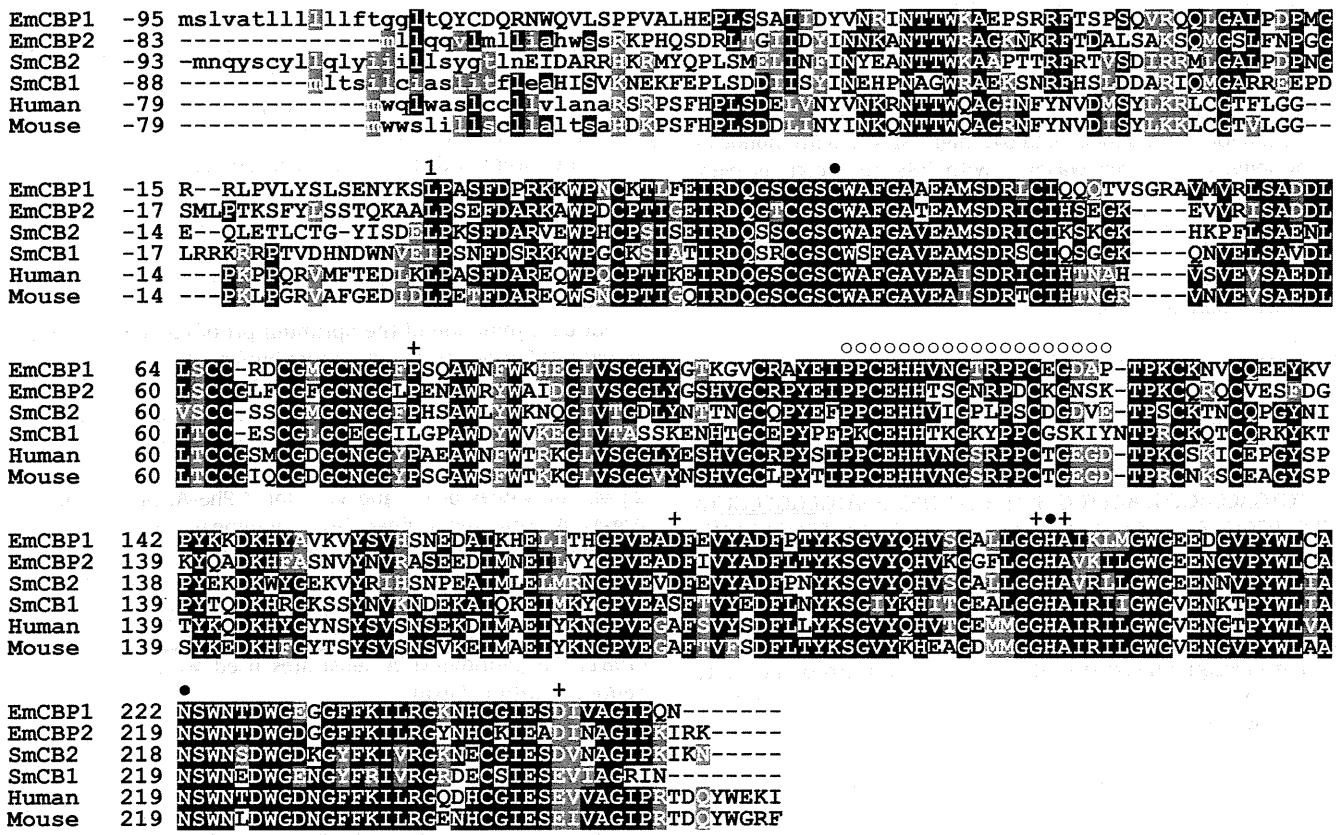

Fig. 1. Comparison of the deduced amino acid sequences of EmCBP1 and EmCBP2 with other cathepsin B enzymes. The alignment was generated using Clustal W server (http://www.ch.embnet.org/software/ClustalW.html) together with BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). Gaps were introduced to maximize the alignment. Conserved residues are highlighted: identical, similar and unrelated residues with black, gray, and white backgrounds. Predicted signal sequence is written in lower case and closed circles (●) represent active site residues. Amino acid residues forming substrate binding pockets (McGrath, 1999) are indicated by plus signs (+), and the occluding loop unique to cathepsin B is indicated by open circles (○). Aligned amino acid sequences are *Schistosoma mansoni* cathepsin B (SmCB2, AJ312106), *Schistosoma mansoni* cathepsin B (SmCB1, AAA29865), human cathepsin B (Human, NP_001899), and mouse cathepsin B (Mouse, 1701299A).

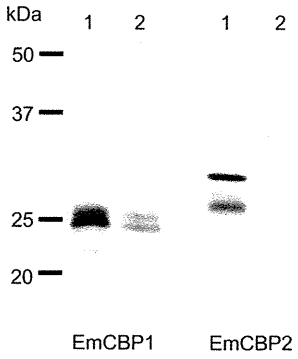

Fig. 2. Immunoblot analyses of *E. multilocularis* metacestodes extracts and ES products. Extracts of *E. multilocularis* metacestodes (lanes 1), and ES products (lanes 2) were probed with anti-EmCBP1 (left) and anti-EmCBP2 (right) monoclonal antibody. Molecular size markers are indicated on the left.

and 25.5 kDa in lysate and ES products, and anti-EmCBP2 monoclonal antibody recognized proteins of 27.0 and 29.9 kDa in lysate and ES products. Isotype-matched negative control monoclonal antibody did not bind to any of these bands (data not shown).

Furthermore, immunohistochemical studies were performed to investigate the localizations of EmCBP1 and EmCBP2 in metacestode. As shown in Fig. 3, the germinal layer, the brood capsule, and the protoscolex were stained. No signals were obtained for the acellular laminated layer of parasite.

3.3. Expression of EmCBP1 and EmCBP2 in yeast

To generate functional peptidases for *in vitro* studies, recombinant EmCBP1(yEmCBP1) and EmCBP2 (yEmCBP2) were expressed in yeast using the *P. pastoris* system and the α-pheromone signal sequence for extracellular secretion. The culture supernatant was collected after 3 days of cultivation and was 20-fold concentrated. The hydrolysis activity of the supernatant treated with and without pepsin in the presence of a reducing agent L-cysteine against Z-Phe-Arg-MCA was tested to determine optimal activation conditions of recombinant enzymes before purification (Fig. 4A). yEmCBP1 was activated at pH 4.5 after 1 h without the pepsin treatment. In contrast, removal of pro-region by pepsin was required for activation of yEmCBP2 (Fig. 4A). The activated recombinant enzyme purified by cation-exchange chromatography as a single peak was analyzed by SDS–PAGE followed by Coomassie Blue staining and immunoblotting (Fig. 4B and C). The purified yEmCBP1 migrated as a broad band between 25 and 50 kDa, and the purified yEmCBP2 migrated as a single band of approximately 27.0 kDa. Treating yEmCBP1 with PNGase F converted the broad band to two bands at 30 and 25.6 kDa (Fig. 4C, lanes 1 and 2), whereas no change in size of yEmCBP2 was observed (Fig. 4C, lanes 3 and 4), which indicated that yEmCBP1 was glycosylated. Analyses using the probe, biotin-Z-Phe-Ala-FMK, able to label specifically active cysteine peptidases revealed that in yEmCBP1 enzymes ranging from 30 to 50 kDa, detected as a 30 kDa band after treated with PNGase F, are active (Fig. 4D, lanes 1 and 2). No active enzyme bands except the 27-kDa enzyme were detected in yEmCBP2 (Fig. 4D, lanes 3 and 4). The labelling of active enzymes with biotin-Phe-Ala-FMK failed by pre-treatment with a cysteine inhibitor, E64 (data not shown).

3.4. Activity of yEmCBP1 and yEmCBP2 against peptidyl-MCA substrates

The substrate specificity of the yEmCBP1 and yEmCBP2 was characterized by using several peptide substrates varying at P2 position (Fig. 5). yEmCBP1 preferred substrates with Phe > Val > Leu at P2 position at an acidic pH optimum of 5.5. Substrate with Pro or Arg at P2 position was also hydrolyzed, but less efficiently. The pH optimum for hydrolyzing substrate with Arg at P2 was shifted to 7.5. yEmCBP2 showed similar features to those of yEmCBP1 except that a marked shifting of the pH optimum for hydrolyzing substrate with Arg at P2 was not observed. yEmCBP2 hydrolyzed peptidyl-MCA substrates more efficiently than yEmCBP1.

Kinetic parameters for hydrolysis of Z-Phe-Arg-MCA (suitable substrate for cathepsin L and B) and Z-Arg-Arg-MCA (cathepsin B-selective substrate) were summarized in Table 1. yEmCBP1 and yEmCBP2 had greater k_{cat}/K_m value for Z-Phe-Arg-MCA over Z-Arg-Arg-MCA. Difference in k_{cat}/K_m values between two substrates for yEmCBP1 and yEmCBP2 was 93- and 137-fold, respectively.

3.5. Inactivation kinetics of yEmCBP1 and yEmCBP2

The kinetics of the pH-induced inactivation of yEmCBP1 and yEmCBP2 were studies at pH 6.5, 7.0, and 7.5, and the reaction between enzymes and substrates (Z-Phe-Arg-MCA) was monitored continuously (Fig. 6 and Table 2). The inactivation of yEmCBP1 at pH 6.5 or 7.0 was not observed during monitoring period and its half-time at pH 7.5 was approximately 318 s. The half-time of yEmCBP2 shortened, from approximately 1689 to 10 s, with increasing pH.

3.6. Degradation of macromolecules by yEmCBP1 and yEmCBP2

To investigate the ability of yEmCBP1 and yEmCBP2 to degrade macromolecules, protein digestion analyses were performed (Fig. 7). In these studies, human IgG and human serum albumin

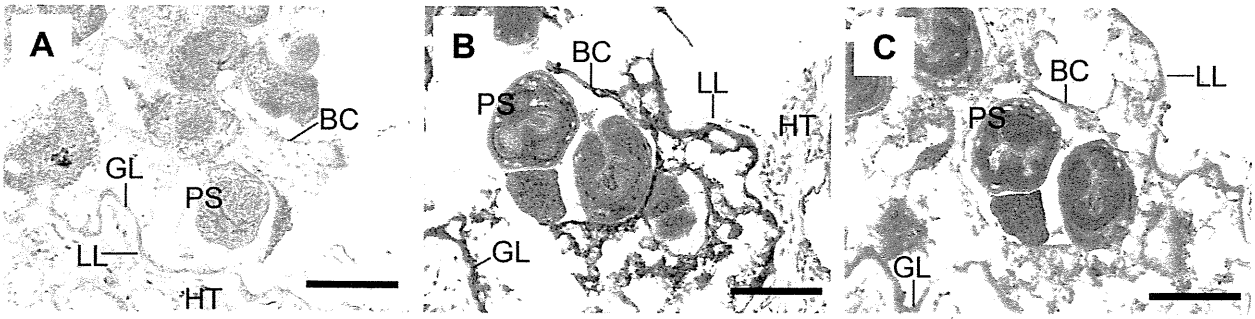

Fig. 3. Immunohistochemical detection of EmCBP1 and EmCBP2 in *E. multilocularis* metacestodes. Parasite tissues (A, B, and C) were isolated and paraffin-sections were produced. The sections were probed with anti-EmCBP1 (B), anti-EmCBP2 (C) and isotype-matched negative control (A) monoclonal antibody. The following structures are indicated: PS, protoscolex; GL, germinal layer; BC, brood capsule; LL, laminated layer; HT, host tissue. Scale bar = 100 μm.

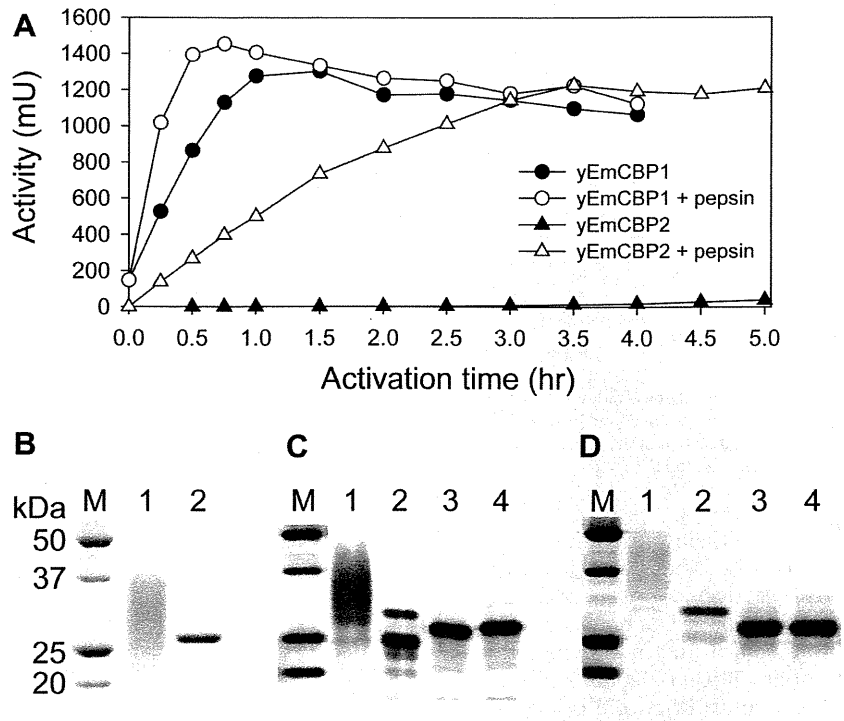

Fig. 4. Expression, purification and active-site labelling of yEmCBP1 and yEmCBP2. (A) Time-dependent activations with and without the pepsin treatment. Aliquots were withdrawn from the incubation mixture at the indicated time points, and the activities were monitored with 50 μM Z-Phe-Arg-MCA. (B) Purified recombinant enzymes were subjected to SDS–PAGE and stained with Coomassie blue. Lane 1, yEmCBP1; lane 2, yEmCBP2. (C) Immunoblot analyses of purified recombinant enzymes before (lanes 1 and 3) and after (lanes 2 and 4) deglycosylation by the treatment with PNGase F. yEmCBP1 (lanes 1 and 2) and yEmCBP2 (lanes 3 and 4) were detected by using monoclonal antibody specific for each protein. (D) Detection of active recombinant enzymes by labelling of purified recombinant proteins with the cysteine peptidase-specific probe, biotin-Phe-Ala-FMK. After labelling, aliquots of recombinant enzymes were treated with (lanes 2 and 4) and without (lanes 1 and 3) PNGase F. yEmCBP1 (lanes 1 and 2) and yEmCBP2 (lanes 3 and 4) were detected with alkaline phosphate-conjugated streptavidin. Molecular size markers are indicated on the left.

as humoral molecules and type I and type IV collagens and fibronectin as extracellular matrix molecules were chosen. All protein substrates used in this study were readily hydrolyzed by yEmCBP1 and yEmCBP2. All degradations of protein substrates were completely inhibited by adding a cysteine peptidase inhibitor, E-64 (data not shown).

4. Discussion

Numerous studies have demonstrated that cysteine peptidases from protozoa, trematode and nematode parasites are involved in various functions including nutrient uptake, disruption of the immune system, invasion and penetration into host tissues, which leads us strongly to consider them as a likely target for the chemotherapy (reviewed by Sajid and McKerrow, 2002; Dalton et al., 2003; Caffrey et al., 2004; Rosenthal, 2004; McKerrow et al., 2006; Robinson et al., 2008; Smooker et al., 2010). By contrast, few characterizations of peptidases including cysteine peptidases of cestodes *E. multilocularis* and *Echinococcus granulosus* have been described (McManus and Barrett, 1985; Marco and Nieto, 1991; Sako et al., 2007). In this study, two cathepsin B-like cysteine peptidases, EmCBP1 and EmCBP2, from *E. multilocularis* metacestode were identified, functionally expressed and characterized.

Sequencing analyses revealed that EmCBP1 and EmCBP2 have a catalytic triad (Cys, His, and Asn) and an oxyanion hole (Menard et al., 1991) those are characteristic features of clan CA family C1 cysteine peptidase. The occluding loop that is responsible for peptidyldipeptidase activity (Musil et al., 1991) and is a feature distinguishing cathepsin B from other cysteine peptidases is also conserved. RT-PCR analyses using EmCBP1 and EmCBP2-specific primer, in addition to the fact that EmCBP1 and EmCBP2 were found in the database of *E. multilocularis* whole genome project (http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-multilocularis.html), demonstrated that the genes obtained were originated from *E. multilocularis*, not from mouse used for preparation of parasite materials (data not shown).

Immunoblot and immunohistochemical experiments demonstrated that EmCBP1 and EmCBP2 were expressed in the germinal layer, the brood capsule and the protoscolex of larva and that some portions of both enzymes were secreted. The sizes, 24.5 and 25.5 kDa, of proteins detected by anti-EmCBP1 monoclonal antibody were smaller than the predicted size, 28.4 kDa. Cathepsin B is synthesized as an inactive 43 kDa pro-form and is processed to a single-chain form (31 kDa) or a two-chain form (heavy chain of 25 kDa and light chain of 5 kDa) to be activated (Towatari et al., 1979). It is possible that EmCBP1 consists of a heavy chain and light chain linking by a disulphide bond, and the protein bands detected by monoclonal antibody might be derived from the heavy chains. Anti-EmCBP2 monoclonal antibody recognized protein of 27.0 and 29.9 kDa in lysate and ES products. The latter protein might be intermediate forms of proenzyme. Determinations of N-terminal amino acid sequences of purified native EmCBP1 and EmCBP2 must be carried out.

The expressions of the active recombinant EmCBP1 and EmCBP2 were conducted by the use of the *P. pastoris* expression system. yEmCBP1 was successfully activated at pH 4.5 and purified. Because of the existence of one N-glycosylation site in mature enzyme, yEmCBP1 was glycosylated and detected as a broad band with 25–50 kDa range in size after purification. Some portion of yEmCBP1, detected as a 25.6 kDa-protein by immunoblot analysis after treated with PNGase F, could not be labelled efficiently with active enzyme-specific probe, biotin-Phe-Ala-FMK, which indi-

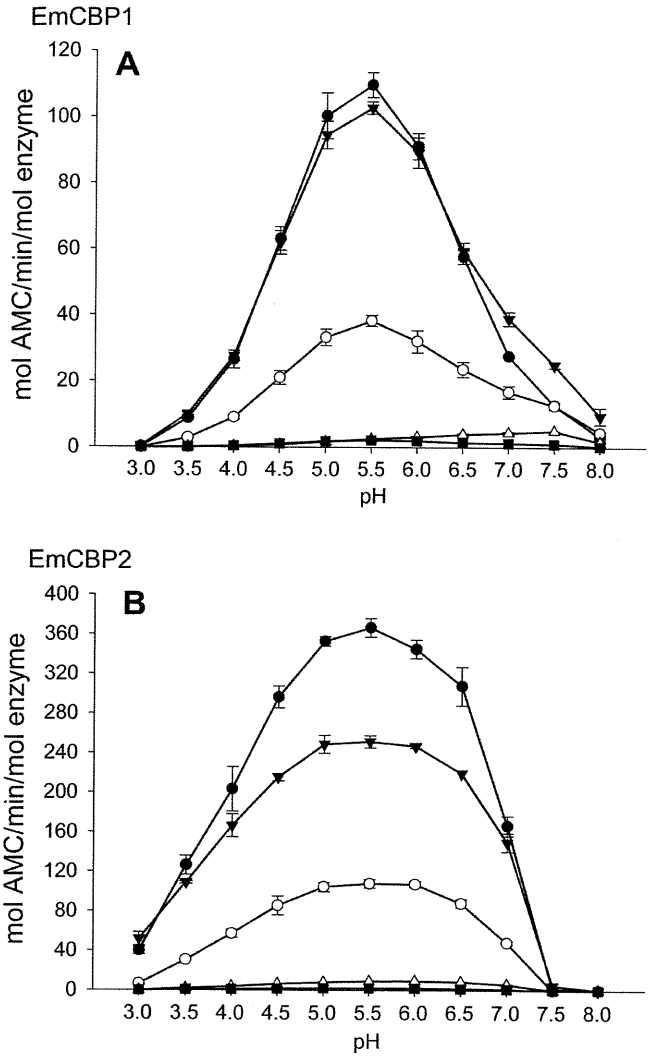

Fig. 5. pH optima and S2 subsite specificity of yEmCBP1 and yEmCBP2. Five substrates, Z-Phe-Arg-MCA (closed circles), Z-Val-Val-Arg-MCA (closed triangles), Z-Leu-Arg-MCA (open circles), Z-Gly-Pro-Arg-MCA (closed squares), and Z-Arg-Arg-MCA (open triangles) were tested at a final concentration of 2 μM. The standard deviation of three experiments is indicated.

cated that they were inactive enzyme. This may be due to miss-folding, the oxidation, or the autodegradation of the mature enzyme during expression and purification. Activation of yEmBP2 was unsuccessful under the same condition of yEmCBP1 activation. Alternative activation condition in the presence of negatively charged glycosaminoglycan with dextran sulfate which facilitates autocatalytic activation (Barlic-Maganja et al., 1998) was tested but resulted in unsuccessful (data not shown). However, active

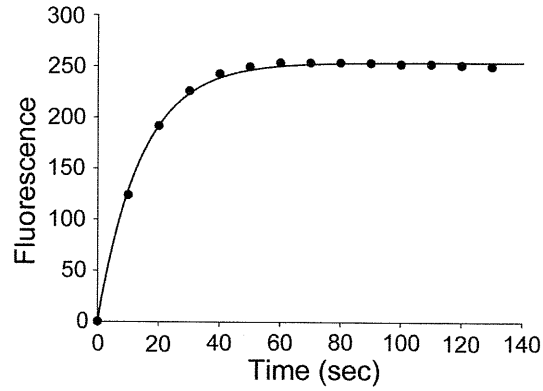

Fig. 6. Progress curve for the inactivation of yEmCBP2 at pH 7.5. Substrate, Z-Phe-Arg-MCA, was tested at a final concentration of 50 μM, and progress of the reaction was monitored continuously by the fluorescence of the released products. The solid line is the theoretical first-order curve calculated using Eq. 1.

Table 2
Effect of pH on the rate of inactivation of yEmCBP1 and yEmCBP2.[a].

	pH	$10^3 \times k_{obs}$ (s^{-1})	$t_{1/2}$ (s)
yEmCBP1	6.5	Nob[b]	Nob
	7.0	Nob	Nob
	7.5	2.18 ± 0.02	317.69 ± 3.40
yEmCBP2	6.5	0.41 ± 0.01	1689.29 ± 61.11
	7.0	6.79 ± 0.48	102.32 ± 7.20
	7.5	70.52 ± 2.23	9.83 ± 0.31

[a] The best estimates for the observed inactivation rate constant, k_{obs}, are given by nonlinear regression analysis. Corresponding half-lives were calculated using following relation ship: $t_{1/2} = \ln 2/k_{obs}$. Inactivation was investigated in the presence of 50 μM Z-Phe-Arg-MCA.
[b] Nob = not observed during incubation.

yEmCBP2 could be obtained by *trans*-processing with pepsin, resulting in a single 27 kDa-protein.

In cysteine peptidases belonging to clan CA, the S2 subsite is substantial substrate-binding pocket for determination of substrate specificity (McGrath, 1999). The substrate specificity of the yEmCBP1 and yEmCBP2 was characterized by the use of several peptide substrates varying at P2 position. Both enzymes preferred substrates with Phe > Val > Leu at P2 position at an acidic pH optimum of 5.5, and cathepsin B-selective substrate Z-Arg-Arg-MCA was less hydrolyzed. Kinetic parameters for hydrolysis of Z-Phe-Arg-MCA and Z-Arg-Arg-MCA revealed that there were 93- and 137-fold-differences in k_{cat}/K_m values for yEmCBP1 and yEmCBP2, respectively. Similar preference has been observed in a cathepsin B isoform (SmCB2) of *Schistosoma mansoni*, not other isoform (SmCB1) (Caffrey et al., 2002). By contrast, the difference reported for mammalian cathepsin B is smaller than 10-fold (Hasnain et al., 1992; Wang et al., 1998). EmCBP1, EmCBP2 and SmCB2 have a negatively charged residue Asp at position 173 (mouse cathepsin B

Table 1
Kinetic parameters for hydrolysis of peptidyl-MCA substrates by yEmCBP1 and yEmCBP2.[a]

	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
yEmCBP1	Z-Phe-Arg-MCA	20.45 ± 1.23	33.23 ± 1.01	1626.71 ± 48.67
	Z-Arg-Arg-MCA	747.67 ± 41.15	13.05 ± 0.63	17.46 ± 0.11
yEmCBP2	Z-Phe-Arg-MCA	27.17 ± 0.82	71.68 ± 1.52	2638.40 ± 23.29
	Z-Arg-Arg-MCA	1124.47 ± 76.15	21.57 ± 1.05	19.19 ± 0.36

[a] The K_m and V_{max} values were calculated by a nonlinear regression analysis of substrate concentration versus peptidase velocity. The k_{cat} values were calculated from V_{max} and the molar concentration of active enzyme titrated with Z-Phe-Arg-MCA and cysteine peptidase inhibitor E-64.

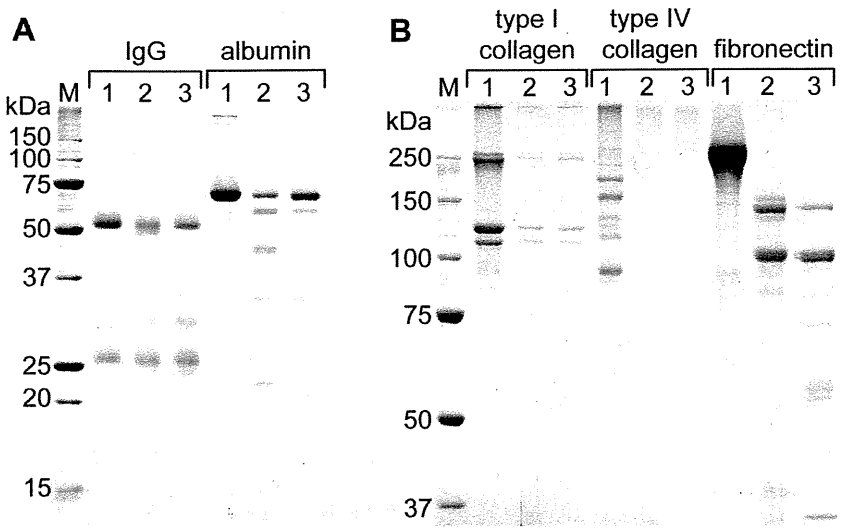

Fig. 7. Degradation of macromolecules by yEmCBP1 and yEmCBP2. Humoral molecules (A) and extracellular matrix molecules (B) were incubated with yEmCBP1 or yEmCBP2 at 37 °C for 4 h at pH 5.5. Protein substrate digests were subjected to SDS–PAGE and degradation products were visualized by Coomassie Blue staining. Each substrate was incubated with none of enzyme (lanes 1), yEmCBP1 (lanes 2), and yEmCBP2 (lanes 3). Molecular size markers are indicated on the left.

numbering) involving in S2 subsite formation, whereas mammalian cathepsin B from human, mouse, rat, and bovine and SmCB1 have an uncharged residue, Ala and Ser, respectively, without other substantial differences. Therefore, the possibility that the negatively charged residue Asp participates in the substrate preference is raised. Further analyses by using recombinant enzymes in which Asp is replaced with Ala are necessary to confirm this possibility.

The facts that at acidic condition yEmCBP1 and yEmCBP2 were stable, active and had broad specificity against host proteins including immunoglobulin, albumin, collagens, and fibronectin, suggested that these enzymes are lysosomal enzymes and are involved in protein digestion for parasites' nutrition. Additionally, the possibility that yEmCBP1 and yEmCBP2 act as extracellular enzymes was raised since both enzymes were detected in ES products. However, yEmCBP1 and yEmCBP2, especially latter, were unstable at neutral or slightly alkaline pH close to physiological pH similarly to mammal papain-like cysteine peptidases except for cathepsin S (Turk et al., 2000). Since the echinococcal cyst fluid has a neutral pH, EmCBP1 and EmCBP2 secreted into cyst fluid might lose their enzymatic activity. In contrast, EmCBP1 and EmCBP2 secreted outside of parasite cysts might not lose their enzymatic activity, because it is known that the host inflammatory responses can lead to tissue acidification (Kellum et al., 2004) and cathepsin B secreted from some kind of tumor cells becomes stable at alkaline pH by interacting with heparin and heparan sulfate (Almeida et al., 2001; Roshy et al., 2003). The activities of cysteine peptidases secreted into host tissues to degrade extracellular matrix molecules, e.g., collagen and fibronectin, have been described in several nematode and trematode parasites (Berasain et al., 1997; Rhoads and Fetterer, 1997; Smooker et al., 2010), and these characters seem to be implicated in migration of parasite through host tissues. Because the larva of *E. multilocularis* infiltrates and proliferates indefinitely by exogenous budding of the cellular germinal layer (Thompson, 1995), the ability of EmCBP1 and EmCBP2 to degrade extracellular matrix molecules might be involved in pathogenesis of *E. multilocularis*. Inhibition analyses of EmCBP1 and EmCBP2 by specific inhibitors or genetic knock out of enzymes would be needed to confirm such a role of peptidases *in vivo*.

In conclusion, we identified and characterized two novel cathepsin B-like peptidases from *E. multilocularis* metacestodes. The enzymes may play a primary role in protein digestion for parasites' nutrition. Thus, the inactivation of these enzymes may impair the survival of the parasite in the host. Further studies are needed to provide a greater understanding of the biological significance of EmCBP1 and EmCBP2 in parasite–host interactions.

Acknowledgements

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PLoS NEGLECTED TROPICAL DISEASES

Viewpoints

Cystic Echinococcosis: Chronic, Complex, and Still Neglected

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The Overall Scene

Cystic echinococcosis (CE), an infection with the larval form of the dog tapeworm *Echinococcus granulosus*, still causes serious lung and liver disease with a worldwide geographical distribution. This parasitic infection is preventable, eliminable, and treatable—in theory. The biological cycle can be attacked at various points: regular dog deworming, controlled sheep slaughtering, vaccination of the intermediate (sheep) animal host, and possibly in the future, vaccination of the definitive (dog) animal host (Figure 1). However, breaking the cycle in practice is difficult and requires long-lasting efforts. Control programs are expensive to set up and sustain. With the currently available options, a period of 20 years is needed to reach elimination, a goal that, unsurprisingly, has only been reached in rich countries [1].

At the current pace of control, patients suffering from CE will be seen for many decades to come. CE disease is chronic, complex, and neglected [2–4]. It is still poorly understood, and recommendations for diagnosis and treatment have not progressed beyond expert opinions and are not necessarily adopted by clinicians because of lack of grade I evidence.

The critical issues are:

(1) CE may develop silently over years and even decades until it surfaces with signs and symptoms or as a chance finding on an ultrasound (US) scan or chest X-rays requested for unrelated reasons. Clinical manifestations may mean that the cyst is already complicated, e.g., ruptured into the biliary or bronchial tree, secondarily infected with bacteria, or leaking and causing allergic reactions if not anaphylactic shock.

(2) Screening large samples of populations to detect asymptomatic cases is expensive. As with all screening procedures, ethical issues arise: do all patients in whom cysts are found require treatment? Is the treatment which we then offer well established and safe? And is it available at all? Screening projects in endemic areas are often inadequately prepared, as the clinical management is not provided locally for those who are found positive.

Problems start with the screening tool. With the exception of liver US, the available methods are far from satisfactory. In regards to serology, the sensitivity and specificity of several antigens have been well defined [5,6], but available assays still lack standardization, sensitivity, and specificity [7]. Controversies on the usefulness for clinical diagnosis and screening remain unresolved [8]. Serodiagnostic performance depends on several factors, such as cyst location, cyst stage, and even cyst size, but these and other variables have not been thoroughly assessed to date.

Ultrasound is an indispensable tool, but will likely miss very small cysts, and its efficacy is mostly restricted to intraabdominal organs. Additionally, some cyst stages may be difficult to distinguish from nonparasitic cysts, which are common. The problem continues when an echinococcal cyst has been diagnosed. In settings where health care facilities are several days of travel away from the rural areas where patients live and work, and as long as we have doubts on what the natural evolution of their cysts will be, clinical decision making is difficult. It has to be done in each case individually based on current standards, clinicians' experience, and local technical possibilities, supported by embarrassingly poor evidence.

(3) Not all CE patients are similar, even at a population level. Broadly speaking, there are two defined groups of patients, each with a different set of problems: mainly asymptomatic patients (detected in screening programs or by chance), or clinically apparent cases (mostly patients with complicated cysts).

(a) Patients with cysts detected during screening activities or as a chance finding. They mostly receive the treatment with which the attending clinician is familiar. This is not necessarily the best option relative to the cyst stage and clinical situation of the patient. Preliminary results from a survey on knowledge, attitudes, and practices regarding clinical management of CE in European, North African, and Middle Eastern countries yielded alarming results [9]. Patients may be put at risk of interventions that may be completely

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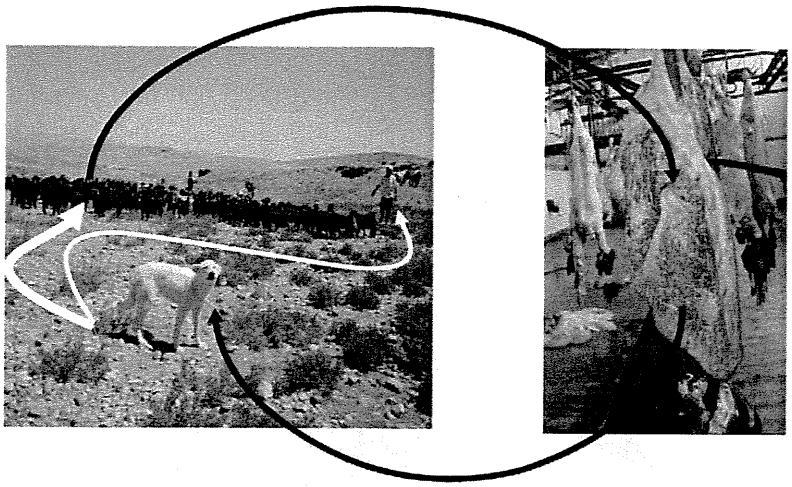

Figure 1. Life cycle of *Echinococcus granulosus* in a community of the Middle Atlas region, Morocco. (We thank M. Kachani, College of Veterinary Medicine, Western University of Health Sciences, for the pictures.)
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unnecessary. This certainly applies to a sizeable number of cysts that have become inactive and do not cause any symptoms or complications.

A significant proportion of cysts stop growing and follow a path to spontaneous involution. Long-term follow-up suggests that these cysts and the patients harbouring them should be left alone. This is an appealing perspective for patients and health services, if evidence can be gathered in its support. CE4 and CE5 cysts appear to be very good candidates for this approach if they do not compromise any vital structures. It is, however, unclear if and under which circumstances this concept can be extended to other cyst types.

(b) Patients developing complications. Successful management depends on equipment, skills, and quality of available health services. The most common complications are biliary obstruction with or without cholangitis, bronchial obstruction, bacterial infection of the cyst cavity with abscess formation, rupture with anaphylactic reactions that range from mild to lethal anaphylactic shock, secondary echinococcosis (growth of new cysts caused by seeding of protoscolices, generally in a cavity such as the peritoneal space) following spillage of fluid from a cyst that ruptured either spontaneously or because of a therapeutic maneuver, and impaired function of organs and blood vessels compressed by growing adjacent cysts (Figure 2). In most endemic countries, the required setup is only met in major cities a long way off from where patients experiencing complications live.

What Is Available Today to Diagnose and Treat CE Patients?

Ultrasound is well established as a tool to diagnose, stage, and follow up CE cysts in the liver and other locations. Gharbi and colleagues developed the first widely adopted US classification in 1981 [10]. Other classifications were subsequently produced but were not as widely used. In 1994, the World Health Organization (WHO)-Informal Working Group started developing an international standardised US classification that could be universally applied to replace the plethora of classifications previously used (Figure 3) [11]. Even with all the obvious advantages of a standardised classification, some important issues still need to be resolved, one being the right sequence of cyst stages seen as the effect of natural or treatment-induced involution. A recent assessment of metabolic profiles of cyst stages with high-field proton magnetic resonance spectroscopy (^{1}H MRS) has shown that the WHO IWGE classification of active, inactive, and transitional stages is perfectly in line with the metabolic activity profiles of the cysts, with the exception of CE3b, which appears vigorously active in ^{1}H MRS, a finding that corresponds well with clinical experience [12]. US has been confirmed as an invaluable tool to assess cysts both with respect to viability and potential complications (Figure 2).

There are basically four management options: surgery, percutaneous sterilization techniques, anti-parasitic treatment, and observation ("watch & wait"). Their individual roles were recently reviewed [2–4]. Each of the four strategies certainly has its place, but the specific places and boundaries are still not well defined.

Surgery, the oldest form of treatment, keeps its place in most of the complicated forms of the disease. There is some competition between surgery and percutaneous approaches, in particular modified catheterization techniques, to be resolved, but this comparison requires carefully designed studies and cannot be decided on the basis of exclusively non-comparative small clinical studies, which are the only ones currently available.

Proponents of classical PAIR (punction, aspiration, injection, reaspiration) [13] have lost a bit of their enthusiasm after realizing that some cyst stages, such as CE2 and CE3b, are quite tedious to needle with too many compartments to be individually approached. But most importantly, these stages tend to relapse after PAIR. It remains to be seen whether large modified catheterization techniques can substitute for PAIR in these stages.

Over the past decade, several studies have been published suggesting that medical therapy (mebendazole, albendazole) could be an alternative to invasive treatment options in patients with uncomplicated cysts, broadening the indication for medical treatment over the years. The individual studies were all small and heterogeneity precluded appropriate meta-analysis. A recently published pooled analysis of individual patient data collected from six treatment centres suggests that the overall efficacy of benzimidazoles has been overrated [14]. Clinical trials stratified by cyst stage are needed to define the place of anti-parasitic treatment in the treatment of CE since it appears that it works better in some cyst stages (e.g., small CE1 cysts) than in others. The rate and nature of side effects of prolonged application of benzimidazole also deserves to be investigated more rigorously. Other anthelmintics, old and new (praziquantel, nitazoxanide), and combinations of anthelmintics (e.g., albendazole plus praziquantel) need to be properly investigated, too.

Though so far not systematically studied, experience with leaving certain cysts completely alone and only following them up over years, points to a fourth managing option, watch & wait. Apart from being biologically plausible, long term follow-up of patients with CE4 and CE5 cysts in anatomically silent corners of the body looks good. This holds great promise for patients in whom cysts have reached this

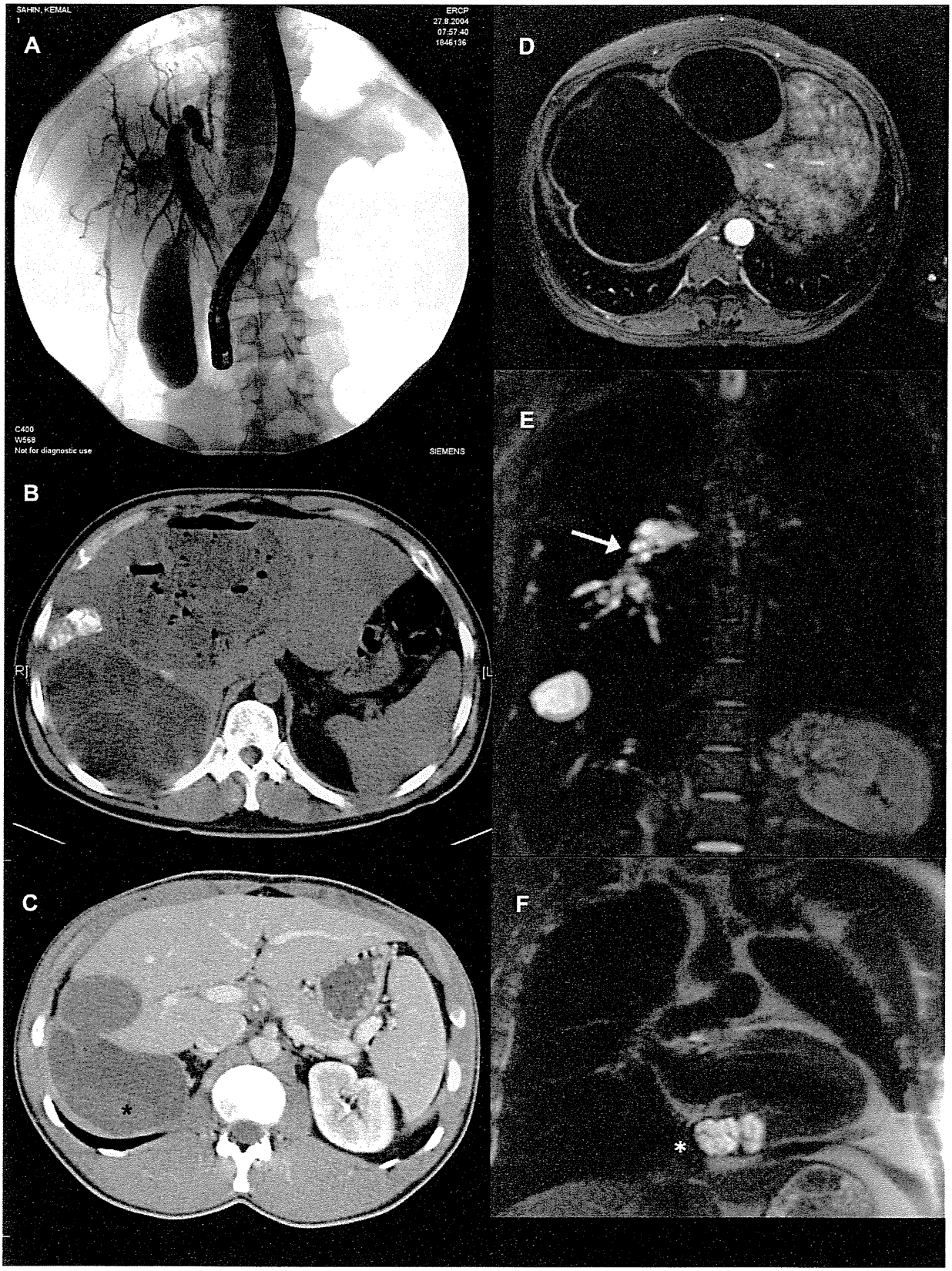
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Figure 2. Severe and life threatening complications of CE. (A) Biliary obstruction/obstructive cholangitis due to biliary fistulas. (B) Liver abscess formation due to secondary bacterial infection of cysts. (C) Cyst rupture (*) followed by anaphylaxis and secondary echinococcosis. (D) Cysts exerting pressure on vital neighbouring structures (e.g., liver veins resulting in Budd-Chiari Syndrome). (E) Embolism of the right pulmonary artery (arrow) caused by cardiac CE and vascular invasion. (F) CE infestation of the posterior wall of the left heart replacing the myocardial layer at the base of the heart. (We thank W. Hosch, Department of Radiology, and A. Stiehl, Department of Gastroenterology, University Hospital Heidelberg, for the images.).
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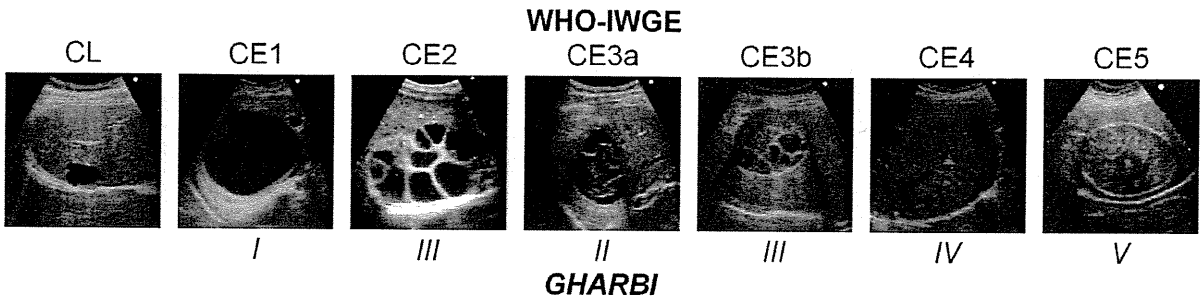

Figure 3. Comparison of Gharbi's and WHO-IWGE ultrasound classifications of CE cysts. CL, as a potentially parasitic cyst, was not in Gharbi's classification and needs to be differentiated from non-parasitic cysts. This may also happen with CE1 cysts, when the double layer sign is not evident. Also, WHO CE3b had not been explicitly described by Gharbi but could likely be classified as Type III.
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stage and needs to be urgently systematically studied.

Reasons for Arrested Progress in CE

Difficult, chronic diseases with a low case fatality rate clustering in poor rural areas are particularly "unattractive" to researchers and funders who depend on quick results to maintain the momentum of their activities. CE shares this fate with other communicable diseases, such as neurocysticercosis and Buruli disease. Health services also turn a blind eye on them since they plainly lack the means to manage patients with complex diseases such as CE appropriately. This is reflected in the low attention national and international institutions are paying to CE despite its substantial global burden, which is estimated at over 1 million DALYs per year [15,16]. Additionally, due to its global distribution pattern, CE is not taking advantage of the attention that is being paid to "tropical" diseases. Interestingly, CE never made it to the list of the "TDR diseases" (from the WHO Special Programme for Research and Training in Tropical Diseases). The scarcity of resources and lack of momentum leads research to develop in niches with research communities too small to plan and conduct projects on a scale that allows conclusive answering of the relevant questions on efficacy, effectiveness, adverse reactions, and costs of a given treatment in comparison to other options. Currently available data arise from a multitude of small underpowered studies carried out over years, leading to contradicting results and recommendations, and, consequently, to controversies and difficulties (e.g., randomization) when planning appropriately designed clinical trials.

What Do We Need to Improve CE Management in the Short Term?

Here is a most clinically neglected parasitic disease that urgently needs attention. A valuable tool for diagnosing, staging, and following up patients, ultrasound, is readily available. Four management procedures, surgery, percutaneous sterilization techniques, anti-parasitic treatment, and watch & wait, have "evolved" over decades, and been recently summarized [4], but without adequate comparative evaluation of efficacy, effectiveness, rate of adverse events, relapse rates, and cost. Clinical decision making is on even shakier ground for extrahepatic and extrapulmonary locations, which are rarer (see [4] for a list of extrahepatic and extrapulmonary locations with related treatments), and numbers needed to build comparative trials hard to come by. There is an obligation to put at least what we have on an appropriate evidence base by conducting comparative clinical trials at the scale and quality that allow answering these important questions. As one of the expected results, clear criteria for the watch & wait option alone might already save a substantial proportion of patients from unnecessary interventions and save health services money. Difficult chronic diseases clustering in poor rural areas need intelligent, creative approaches, and this one urgently needs operational research incorporating the particularities of resource-poor settings into consideration.

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