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Characterization of *em*Y162 encoding an immunogenic protein cloned from an adult worm-specific cDNA library of *Echinococcus multilocularis*

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Abstract

A cDNA library based on mRNA from adult worms of *Echinococcus multilocularis* was constructed. One cDNA clone, *em*Y162, was isolated from this cDNA library. The putative protein from *em*Y162 cDNA consists of 153 amino acids and has a predicted molecular weight of 17.0 kDa. The amino acid sequences of EMY162 are predicted to be a hydrophobic N-terminus conserving a secretory signal, and a hydrophobic C-terminus encoding a transmembrane domain or glycosyl-phosphatylinositol membrane anchor, and to have single fibronectin type III-like domain. In addition, it was shown that the *emY162* gene (1738 bp) in the *E. multilocularis* genome DNA consists of three exons and two introns, and that *em*Y162 is expressed in all four stages (protoscoleces, cultured metacestodes, immature adult worms and mature adult worms). Moreover, immunity to recombinant EMY162, which comprises the fibronectin type III-like domain on the EMY162 protein, was examined. Immune responses to the recombinant EMY162 were studied by using serum from dogs infected with *E. multilocularis*. Strong IgG immune responses were detected in Western blots.

Keywords: *Echinococcus multilocularis*; Adult worm; cDNA library; *em*Y162 cDNA; Immunogenic protein; Recombinant EMY162 antigen

1. Introduction

Echinococcus multilocularis is a cestode parasite [1]. The larva, metacestode, develops in several mammalian intermediate host species, while the adult tapeworms develop mainly in dogs and foxes (definitive hosts). The life cycle of *E. multilocularis* generally occurs in foxes and rodents as intermediate hosts. Humans can be infected by accidental ingestion of the parasite eggs from an infected fox, or occasionally from infected dogs or cats. Invasion by *E. multilocularis* leads to destruction of the liver, and to damage of other organs via metastases [2]. Infection in humans causes alveolar hydatid disease [1]. Although human infection is uncommon in many countries, the disease has a high prevalence in the European latitudes north of the Alps [3] and south Gansu in China [4]. Similarly, the disease is endemic in the island of Hokkaido, Japan. In these areas the disease is a significant public health problem.

The basis of the strategy to reduce the risk of human infection is to break the cycle of transmission and avoid the production of infectious eggs. Protection against infection based on this strategy has been already achieved in the genus *Taenia*, which causes cysticercosis. Protective vaccination with either recombinant proteins or peptide epitopes of 45W, TO16, TO18 and TSA18 proteins had a high efficacy against *T. ovis* infection in sheep and *T. saginata* infection in cattle [5–8]. In addition, a recombinant vaccine has been developed for use in the control of cystic hydatid disease caused by *E. granulosus*. Lightowlers et al. [9] have demonstrated that the recombinant EG95 is a highly effective vaccine to prevent infection with *E. granulosus* in sheep in Argentina, Australia and New Zealand. These experimental results indicate that the prevention of the disease by vaccination, with a high degree of protective immunity against parasites, is possible. Vaccination of foxes and/or dogs (the definitive hosts) is postulated to be one of the most effective

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measures to control the spread of *E. multilocularis*. Gauci et al. [10] reported that EM95 recombinant protein induced significant levels of protection in mice (intermediate host) infected with *E. multilocularis* eggs. However, no information is available about the vaccination of dogs to prevent infection with *E. multilocularis* due to its biohazardous nature, and few studies have been conducted on immunity to *Echinococcus* infections in definitive hosts.

Meanwhile, many vaccine candidate proteins have been discovered that are secretory and anchored on the surface of parasites. They usually possess N-terminal hydrophobic signal peptides and C-terminal hydrophobic trans-membrane domains, and are involved in host–parasite interactions. These secreted and trans-membrane proteins participate in parasite functions including penetration and establishment in host tissues and modulation of host immune responses. These secretory proteins are candidates for development of an *E. multilocularis* vaccine, or for diagnostic use in canines. cDNAs encoding secreted and trans-membrane proteins from a parasite cDNA library have been isolated [11,12].

We attempted to clone cDNA of secretory proteins involved in immune defense systems in order to use them in the control of alveolar hydatid disease. One of the cDNA clone, *em*Y162, proved to be the first new secretory protein that acted as an antigen recognized by the canine immune system. In this paper, we report the analysis of *em*Y162 cloned from an adult worm-specific cDNA library, and the immune responses to recombinant EMY162 with the serum from *E. multilocularis*-infected dogs.

2. Materials and methods

2.1. Materials

E. multilocularis (Nemuro strain) was obtained from a dog–cotton rat life cycle maintained at the Hokkaido Institute of Public Health. Protoscoleces were taken from a cotton rat infected with the Nemuro strain and washed with PBS. Immature adult worms were collected on day 20 post-infection from a dog experimentally infected with *E. multilocularis* protoscoleces. Mature adult worms were also collected on day 60 post-infection. The worms were first released from the intestinal contents by soaking in PBS to remove canine intestinal mucus, then rinsed several times in PBS. The cultured metacestodes were obtained by an *in vitro* culture system basically following Hemphill and Gottstein [13] and Spiliotis et al. [14]. After washing with PBS, all parasite materials were immediately soaked in RNA Later (Ambion, Inc.) and stored in liquid nitrogen. In addition, 1×10^5 of *E. multilocularis* protoscoleces in 2 ml of PBS were used for oral infection. Five Beagle dogs (male, 16 months old) were bled at 40 days after infection, and sera were stored individually at −30 °C until examined for IgG response. The sera of 5 uninfected-Beagle dogs as the control were treated similarly. All experiments were performed in a specially designed safety facility, the Hokkaido Institute of Public Health (biosafety level 3).

2.2. cDNA library construction

Total RNAs from immature and mature adult worms were isolated using Isogen (Nippon Gene) according to the manufacturer's instructions. About 3 ml of parasite material was used for extracting total RNA. mRNAs in the total RNA were prepared using an Oligotex-dT30 (Super) mRNA Purification Kit (Takara Bio), and then reverse transcribed to cDNA using the SMART cDNA Library Construction Kit (BD Biosciences) for first-strand cDNA synthesis in a total reaction volume of 20 μl. The cDNA was inserted into the *Sfi*I site of the vector. The vector was used to prepare a cDNA library in bacteriophage λTriplEx2 (BD Biosciences) according to the manufacturer's recommendations. The library was screened by random cloning. Five hundred forty clones were identified from about 400,000 recombinant phages. The clone, designated *em*Y162 was excised from pTriplEx2 in host bacteria XL-Blue according to the manufacturer's recommendations (BD Biosciences).

2.3. Isolation of emY162 clone from cDNA library

The *em*Y162 clone was isolated as follows. Recombinant bacteriophages of the cDNA library were infected with XL1-Blue bacteria. After treatment for 15 min at 37 °C, the infected bacteria were spread on to a LB/$MgSO_4$ plate and then incubated at 37 °C overnight. Single plaques were transferred to SM solution and kept at 4 °C overnight. The SM solution containing the recombinant phage was treated with BM25.8 bacteria. After incubation for 1 h at 31 °C with shaking, the transformed bacteria were spread on a LB–carbenicillin (50 μg/ml) plate and incubated at 31 °C overnight. The cloned bacteria were grown in LB–carbenicillin (50μg/ml) medium at 31 °C overnight. The bacteria were harvested by centrifugation at 2600 rpm. Plasmid DNA was isolated by using QIAGEN Plasmid Tip 20 (Qiagen). The nucleotide sequences of the plasmid were analyzed on a Gene Analyzer, and the *em*Y162 clone was determined.

2.4. Amplification of emY162 gene in genome DNA

Genome DNA was extracted from protoscoleces of *E. multilocularis* using a DNeasy genomic Kit (Qiagen). One μl (1 ng/μl) of the solution was used as the template DNA for PCR. Primers were designed according to the DNA sequence of *em*Y162 compiled by Gene Works sequence analysis software: upstream primer: 5′-ggaagatggtacttcgattctgt-3′, downstream primer: 5′-tgagggcctgtaagttc-caact-3′. Two additional primers (5′-gagctaatagcaaagttg-3′ and 5′-cacgtgaatc-catcggaagt-3′) were also designed to sequence a fragment of *em*Y162 DNA. PCR amplification was carried out by a Gene Amp PCR System 9700 (Applied Biosystems) in 50 μl of reaction mixture with Taq DNA polymerase (Roche Diagnostics). PCR conditions were as follows: 94 °C for 2 min, then 30 cycles of 93 °C for 30 s, 55 °C for 30 s, and 68 °C for 4 min, and finally 68 °C for 7 min. The amplified DNA fragment was purified with TaKaRa Easy Trap v. 2 (Takara Bio) after separation by agarose gel electrophoresis. This was used as the template DNA for the sequencing reactions.

2.5. Detection of emY162 cDNA in four stages

The *em*Y162 cDNA was amplified by reverse transcriptase (RT)-PCR from each of the four stages (protoscoleces, cultured metasestodes, immature adult worms, and mature adult worms) of *E. multilocularis*. Total RNA from the four stages was isolated using an RNeasy Mini Prep Kit (Qiagen) according to the manufacturer's instructions. About 0.1 μg of total RNA was used for RT-PCR amplification of the *em*Y162 cDNA by the SMART cDNA Library Construction Kit (BD Biosciences). The same primers (5′-ggaagatggtacttcgattctgt-3′and 5′-cacgtgaatccatcggaagt-3′) used for amplification of the *emY162* gene in genome DNA were used for RT-PCR. The predicted size of the RT-PCR product was 144 bp. In addition, two primers (5′-gttgtgctatgtggcactcgact-3′ and 5′-caatccagacagagtatttgcgttc-3′) were also designed to amplify a fragment of β-actin cDNA of *E. multilocularis* to monitor the integrity of the RNA from each stage. PCR amplifications were carried out using Gene Amp PCR System 9700 in 50 μl of the reaction mixture with Taq polymerase (Roche Diagnostics). The conditions for PCR were as follows: denaturation at 94 °C for 2 min, then 35 cycles of 93 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, and finally 68 °C for 7 min. Fragments were visualized by ethidium bromide staining after agarose gel electrophoresis.

2.6. Preparation of recombinant EMY162

The *em*Y162 DNA fragment was amplified from *em*Y162-pTriplEx2. An up-stream primer (5′-agatctgtagacccagagctaatag-3′) with a *Bgl*II site and a downstream primer (5′-ctgcaggaatccgccagctctgtca-3′) with a *Pst*I site were designed for amplification of a 360-bp fragment of *em*Y162 DNA. PCR amplification was carried out using the Gene Amp PCR System 9700 in 50 μl of reaction mixture with Taq DNA polymerase (TakaraBio). Amplified DNA was subcloned into a *Bgl*II/*Pst*I-digested ThioHis vector (Invitrogen) and then

transformed into *Escherichia coli* Top10 strain (Invitrogen). The bacterial culture was incubated overnight at 37 °C. After cultivation, recombinant EMY162 was induced with 0.5 mM isopropyl-D-thiogalacto-pyranoside for 4–5 h at 32 °C, and following centrifugation, suspended in B-PER plus a protease inhibitor. The recombinant EMY162 expressed as a fusion protein with ThioHis was treated with ProBond™Affinity Resin (Invitrogen). The fusion protein was eluted with 20 mM sodium phosphate buffer (pH 6.0) containing 500 mM imidazole and 500 mM sodium chloride, and purified by AKTA Explorer (Amersham Biosciences) with a Hiload Superdex 75 pg column equilibrated with 20 mM Tris–HCl buffer (pH 7.8) containing 500 mM sodium chloride. Purified protein was used in Western blotting.

2.7. Detection of IgG response by Western blotting

Western blotting was carried out as follows. Approximately 1 μg of recombinant EMY162 was loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane (Amersham Biosciences), and then treated with PBS containing 10% skim milk and 0.1% Tween 20 for 1 h at room temperature. The recombinant EMY162 protein was detected with diluted canine serum (1:400) at 40 days after infection with *E. multilocularis* and diluted AP-labeled rabbit anti-dog IgG (1:2500), using the BCIP/NBT Immuno-detection Kit (PerkinElmer) according to the manufacturer's instructions.

2.8. Analysis of DNA and protein sequences

Plasmid DNA containing the parasite cDNA was prepared using a DNA Purification Kit (Qiagen) and used as a template in DNA sequencing reactions. Sequencing was performed using the 3130*xl* Gene Analyzer with a Dye-terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequencing of the *emY162* gene was also performed by the same means. DNA and protein sequences were compiled using Gene Works (v. 2.5.1, Teijin) sequence analysis software. The sequence was aligned to other gene sequences available in a Basic Local Alignment Search Tool (BLAST) search of GenBank databases.

3. Results and discussion

cDNA library was constructed from immature adult *E. multilocularis* worms. In the cloning experiment, 540 cDNA clones were isolated. Their nucleotide and predicted amino acid sequences were aligned to other sequences available in GenBank databases. The alignment hit one protein sequence relating to secretory proteins containing EG95 and EM95 proteins that act as antigen.

The *em*Y162 cDNA (GenBank accession number: AB303298) cloned from the immature adult worm cDNA library of *E. multilocularis* comprised 776 nucleotides with an open reading frame of 462 bases. Comparison of nucleotide alignments showing the presence of ATG at the 5′ end and TGA (stop codon) at the 3′ end indicated that this cDNA represents a complete copy of the mRNA of the *emY162* gene. This cDNA sequence does not appear to be a copy of any other gene sequence available in a BLAST search.

The deduced amino acid sequence of *em*Y162 cDNA showed similarity to the previously described antigenic secretory proteins EM95 and EG95-1 (30% and 36%, respectively) [10,15]. When a BLAST search was also conducted for homology to antigenic proteins of *T. ovis* and *T. saginata* parasites, the EMY162 protein showed less than 30% identity to all antigens [5,7,16]. In addition, the amino acid sequence of EMY162 showed some similarity to a part of tenascin, collagen-like protein, protein tyrosine phosphatase (receptor type), and fibronectin 1, according to a BLAST search of GenBank databases. Comparison with amino acid sequences of the cDNAs that

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AAGATGGTAC TTCGATTCTG TCTTATTTTA CTGGCAACTT CAGTTATCGC TGAGGAAGTC GGGGTAGACC CAGGTAAGAT ACCTTTAAAA TTCAACTTGC   100
   M  V  L   R  F  C   L  I  L   L  A  T  S   V  I  A   E  E  V   G  V  D  P
ATCAGCATAC AATTTAAATT ATATAACAAG CTTGTATTCA TCTGCGTATG CATAGTATGT TGTGGTGGAT GCTTTTCAAC TCGTTAGTAG TTAACAGATG   200

ACCAGTTGGC TACTACCACT CATTTGATAA TATGTCACTG TCAAATCTAA AATGACGATT TTTAGCAGAA TCATTGACTC CATATTCCTA CTGCTGTCCT   300

CCTTCTGTAT CAGTTCGGGT AGATATTTGA GTCATCTGCG AAGTGTCATC CACGGGTAGG GAAATGCTTC AAAAATACAC CCTCACTTAG AAATGTAGTC   400

CCAAACACTG ATAGTAGACT GATTCACTGC ACCTCACGAC TACTTATTAT TGCACACAAT TTGTGTACCC TGCACTTGAT AGAACATTGC CTTGCTGATA   500

TTTCGCCTCA TTGTGTTCAC TCGCATAGTA CACTCCCACT TAAACAGCTC GTAAGGTAAA AGGCCTTCGG TATCTGGTCG CACCTACACA TCACCTCGTC   600

ACTGCGTGCA ACTTCAGCTG TAGCAAACCC ACCACTGACA CTATAACTCT CCGAAATCAG GATGTAGTGA CATCCATTTA CCTGCATGAT GGGTCCAGGA   700

TGACAGGAAG AAGTGCCTTG ACACACTTCA TATTGTTGGA ACACAATCCA CAAACTTAAC TTAAGCCTCA TGTTTAACCT CTACCCAAG CATTCCGCTT   800

AATTTCGCTA AAAGCTTAGC CAAGAAGTGA CTGGAAAATT GGCTGAAATT GTTAATTTTG CAATGGGTTT GCTCCGGAAA AAAGGTTGCG CACGCGTGTG   900

TACACATGAC TGGCTATTGC GCATCAACGG ATGACACTCA GCAATTCTTA CCTCTGGTGG AACATACGGG TTAATTACTC CATCGTTTAG GGGAACACAG  1000

CAAAGTCCTG TTATCCACTG CCATCATTGT CATTATGTTT GGATAGAGGG TGCAACATTT CTGCCATTAA ATGGAATGGA ATCAACAAAA CATTATTGTT  1100

GAACAACGAA TTTTTCCGAT TCGTTTTTGC AGAGCTAATA GCAAAGTTGA CAAAGAAACT ACACAGACCA CTGCCAGAAC ACTTCCGATG GATTCACGTG  1200
                                E  L  I   A  K  L  T   K  K  L   H  R  P   L  P  E  H   F  R  W   I  H  V
GGTTCCCGCT CCCTTGAATT GGGTTGGAAT GCCACTGGTT TAGCCAATCT CCACGCGGAC CACATTAAAC TGACTGCAAA CCTTTATACA ACTTACGTTT  1300
G  S  R  S   L  E  L   G  W  N   A  T  G  L   A  N  L   H  A  D   H  I  K  L   T  A  N   L  Y  T   T  Y  V  S
CATTCAGGTA CAGAAATGTT CCTATCGAAC GTCAGAAACT CACTCTTGAG GGACTAAAGC CCAGTACATT CTACGAAGTG GTTGTGCAAG CACTCAAAGG  1400
  F  R  Y   R  N  V   P  I  E  R   Q  K  L   T  L  E   G  L  K  P   S  T  F   Y  E  V   V  V  Q  A   L  K  G
GGATTCCGAA GTTTATAAAT ACACTGGATT TATTAGAACA CTGGCTCCAG GTAAGCTAGC ATGCGATAGT AATGTGAACT TCACACCGAG GATGGATGCC  1500
 D  S  E   V  Y  K  Y   T  G  F   I  R  T   L  A  P
CACCAAACTT CACCTTTGGC GGCAGTTCCT GTGTTATTAC ACTAGACCAC AAGTTAGACG AACCACAAGG CGTCAGTAGC TAATGCTCAA ATTTTGCAAA  1600

GTTTGCCGTT GAGTGGCACT GGTGTGCTGC GTAACGACAC TGACAGAGTG TCATTTTCGC ATTGCAGGGG AAGATGGCGC TGACAGAGCT GGCGGATGCG  1700
                                                                  G  E   D  G  A   D  R  A   G  G  C  A
CCCTAATTTT TGCAATGGCT GGGCTCCTAT TACTTACTTG AGCCTTCCCG TAAGCCAATG AAGGTGGTCA ACTGTTGCAT TAGTTGGAAC TTACAGGCCC  1800
  L  I  F   A  M  A   G  L  L  L   L  T  .
TCA                                                                                                          1803
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Fig. 1. Nucleotide sequences (1803 bp) of *emY162* gene in *Echinococcus multilocularis*. Predicted amino acid sequence of the encoded protein is shown below the DNA sequence.

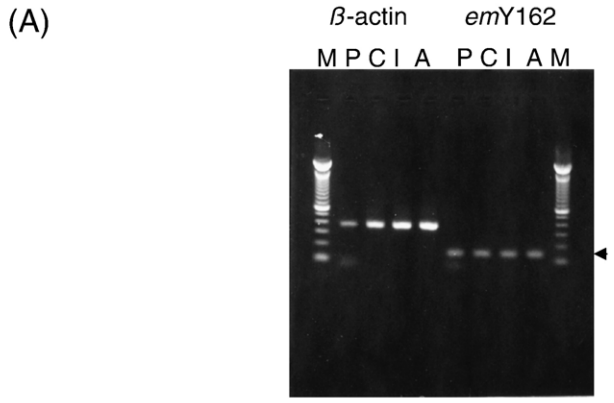
produce the known secretory protein in parasites of the genus *Taenia* did not identify significant homology.

The EMY162 protein consists of 153 amino acids and has a predicted molecular weight of 17.0 kDa. This protein has a relatively high proportion of leucine residues (16%) and one putative N-linked glycosylation site at amino acid position 83. The predicted structure from the amino acid sequence of EMY162 consists of a hydrophobic N-terminus (amino acids 1 to 16) predicted to be a secretory signal, a hydrophobic C-terminus encoding a trans-membrane domain or glycosyl-phosphatylinositol membrane anchor (amino acids 130 to 153), and one fibronectin type III domain (amino acids 84 to 129) [17–19]. Bork and Doolittle [20], Bork et al. [21] and Campbell and Spitzfaden [22] have indicated that proteins with fibronectin type III domains include the immunoglobulin superfamily, cell adhesion, surface receptors and carbohydrate-binding proteins. Analysis of the predicted amino acid sequence has revealed the presence of a conserved motif. The motif defines a fibronectin type III domain, having 40% homology compared to fibronectin sequences available in a BLAST search of GenBank databases.

The *emY162* gene (GenBank accession number: AB303297) in genome DNA was amplified by PCR from the *E. multilocularis* genome DNA. A 1738-bp DNA fragment encodes amino acid sequences of the EMY162 protein. The *em*Y162 nucleotide sequences (1803 bp) are shown in Fig. 1. The *emY162* gene consists of three exons and two introns, with similarities in structure to secretory proteins such as the *em95* gene [10]. The intron splice sites of the *emY162* gene are conserved in comparison with the cDNA. Exons 1, 2 and 3 of the gene and cDNA are identical in the DNA sequence. The lengths of exons 1, 2 and 3 are 70, 318 and 74 bp, respectively. The intron length of the *emY162* gene does not appear to be as conserved as that of the *em95* gene [10]. The lengths of intron 1 and 2 are 1059 and 217 bp, respectively.

The length of exon 1 in the *emY162* gene is similar to that of the *em95* gene, while exons 2 and 3 differ in these genes [10]. The lengths of exon 2 and 3 of *em95* were 306 and 92 bp, respectively [10]. The exon lengths of the *emY162* gene do not appear to be as conserved. In addition, the length of intron 1 of *em*Y162 also differs from the length of intron 1 in *em*95. These analyses clearly indicate that the *em*Y162 isolated from the cDNA library based on mRNA from adult *E. multilocularis* tapeworms codes for a novel secreted transmembrane protein that is different from the gene family of secretory proteins such as em95.

Meanwhile, the same putative size (144 bp) was detected in all four stages examined. The RT-PCR products are shown in Fig. 2(A). The amplified sequence covers exon 1 and a part of the N-terminus in exon 2 of EMY162 protein that includes the



(B)

```
P-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50
C-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50
I-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50
A-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50

P-emY162F1/Ra CGCTGAGGAA GTCGGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
C-emY162F1/Ra CGCTGAGGAA GTCGGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
I-emY162F1/Ra CGCTGAGGAA GTCGGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
A-emY162F1/Ra CGCTGAGGAA GTCGGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100

P-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
C-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
I-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
A-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
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Fig. 2. Agarose gel electropherogram (A) and nucleotide sequences (B) of products amplified by reverse-transcriptase polymerase chain reaction (RT-PCR). (A) Total RNA from protoscoleces (P), cultured metacestodes (C), and immature (I) and mature adult (A) worms of *Echinococcus multilocularis* were used to amplify *em*Y162 cDNA with β-actin cDNA (379 bp) as the control. The position of the amplified *em*Y162 cDNA band of the expected size (144 bp) is indicated by an arrow. Molecular (M) size markers are shown in the left and right lanes. (B) Nucleotide sequences of 144-bp products of protoscoleces (P), cultured metacestodes (C), and immature (I) and mature adult worms (A) amplified by using RT-PCR with the primer pair of F1 and Ra are shown.

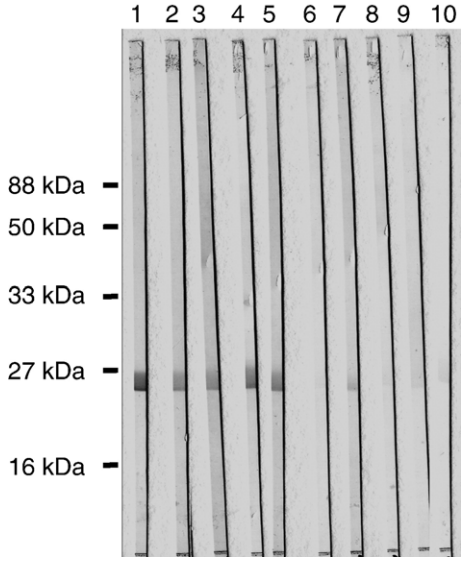

Fig. 3. Immunoblot analysis of recombinant EMY162 antigen using sera of dogs infected with *Echinococcus multilocularis*. Lanes 1–5: sera of dogs infected with the Nemuro strain (40 days after infection); lanes 6–10: sera of uninfected dogs.

peptide sequences (MVLRFCLILLATSVIAEEVGVDPE and LIAKLTKKLQTTLPEHF). The primers used in the RT-PCR analysis, according to the *em*Y162 mRNA sequence, span an intron of 1059 bp in the *emY162* gene. In a comparison of sequence alignment, PCR products from each of the four stages corresponded to positions 1 to 140 in *em*Y162 mRNA. The 144-bp RT-PCR products indicated that they contained cDNA sequences only, without introns. The results of RT-PCR indicated that *em*Y162 is expressed in all four stages. In addition, the nucleotide sequences of the 144-bp RT-PCR products are shown in Fig. 2(B). The sequences were all the same. No difference was detected in the peptide sequences of *em*Y162 mRNA expressed in all four stages.

As shown in Fig. 3, IgG responses to the recombinant EMY162 in dog serum at 40 days after infection were detected by using Western blotting. A blotting band was observed at a predicted molecular weight of 27 kDa, but it was not detected in the serum of the uninfected dogs used as the control. In this experiment, sera of five dogs that were infected with 1×10^5 *E. multilocularis* protoscoleces showed strong IgG response to recombinant EMY162. It is therefore possible that EMY162 could be used as a diagnostic antigen for serological evaluation of canine *E. multilocularis* infection.

Alignment of the amino acid sequences of *em*Y162, *em*95, *eg*95-5 and TSO45w-4B is shown in Fig. 4. The alignment data showed that amino acid differences are evident in the fibronectin type III-like domain and C-terminus, while these genes were 70% similar to amino acid sequences in the N-terminus. The significant differences are an insertion of four amino acids in the fibronectin type III-like domain and a deletion of seven amino acids in the C-terminus. In addition, the amino acid sequences of *em*Y162 were over 70% different in the linear immunogenic regions of *eg*95-5 and *em*95. The linear immunogenic regions of the EM95 and EM95 proteins include the peptide sequences TETPLRKHFNLTPV (peptide 6), SLKAVNPSDPLVYKRQTAKF (peptides 12/13), DIETPRAGKKESTVMTSGSA (peptides 21/22) and SALTSAIAGFVFSC (peptide 24) [23–25]. As shown Fig. 4, the amino acid sequences of the EM95 proteins are very similar to linear immunogenic regions of the EG95-5 protein. However, significant differences existed in the amino acid sequences of EMY162 protein as compared with two EM95 and EG95-5 proteins. In considering the use of EMY162 as a serodiagnostic

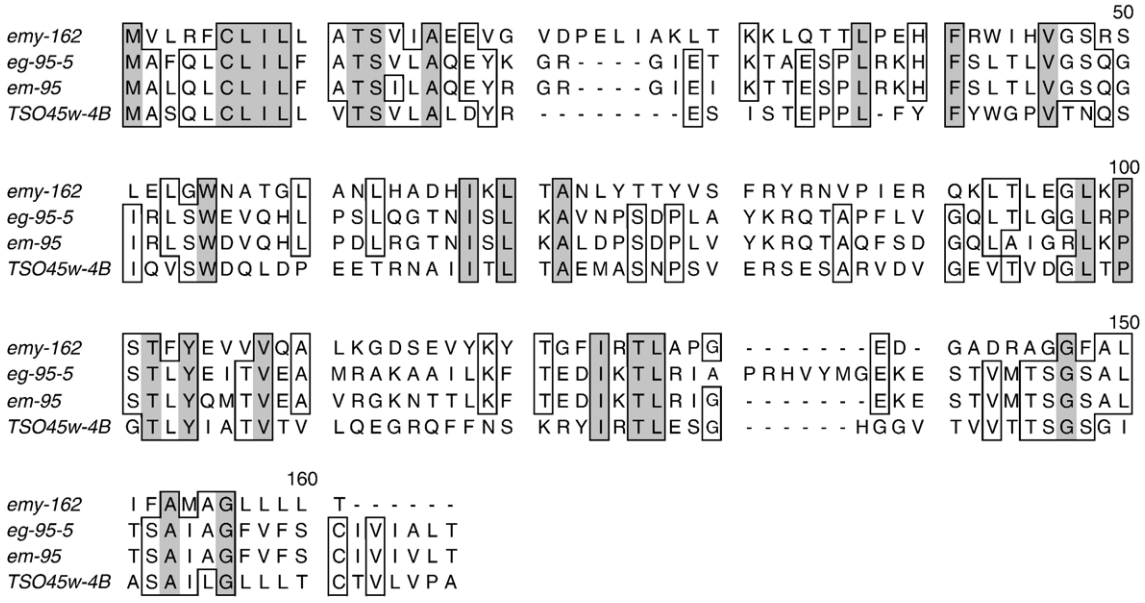

Fig. 4. Alignment of amino acid sequences of *em*Y162 and *em*95 from *Echinococcus multilocularis*, *eg*95-5 from *Echinococcus granulosus* and *TSO*45W-4B from *Taenia solium* (EM95, EG95-5 and TSO45W-4B sequences; GenBank accession numbers AJ420235, AF134378 and AF267119, respectively). The genes of each species have conserved amino acid sequences designated by gray and clear boxes.

tool, it will be essential to characterize host antibody responses, especially the kinetics of the specific antibody response after infection and subsequent chemical deworming, and class and subclass specificity against EMY162. In other parasitic infections in dogs, Deplazes et al. [26] and Nieto et al. [27] demonstrated that the analysis of IgG subsets in parasitized dogs provides evidence of a dichotomous response to infection: IgG2 is associated with asymptomatic protozoan infections and IgG1 is associated with helminth infections and diseases caused by protozoan infection.

Overall, *em*Y162 has features similar to those encoded by oncosphere antigens like *em*95, but does not share significant homology within its sequence. We demonstrated that EMY162 could target both mucosal and systemic immunity in dogs because it is predicted to be a protein with a fibronectin type III-like domain, while the serum of infected dogs showed strong IgG antibody responses to the recombinant EMY162. EMY162 could provide a potential route for the development of a practical vaccine to reduce the level of echinococcosis in canines. Future research will therefore focus on investigating the protective potential of the EMY162 protein or its peptide epitopes against infection with *E. multilocularis* eggs in intermediate and definitive hosts.

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