# Construction and Analysis of a DNA Cassette for Use in DNA-based Immunization: A Potential Application for Producing Antibodies Against *Fasciola gigantica*

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**Abstract**

*Fasciola gigantica*, the tropical liver fluke, is responsible for huge economic losses in the meat industry. Unfortunately, a protective vaccine against fascioliasis is not available. In this study we analyze the use of cathepsin L (CatL), the major secreted proteolytic enzyme of this parasite, for DNA-based immunization in mouse. A chimeric DNA cassette containing the coding sequence for the transmembrane/cytoplasmic (Tm/Cyt) domain of the mouse lysosome-associated membrane protein (LAMP-1) and the CatL encoding sequence of *F. gigantica* was constructed in the mammalian expression vector pcDNA3.1. LAMP-1 was selected as a co-stimulatory for this study as its combination with parasite/virus-specific sequences had provoked strong antigen-specific immune responses in mice in previous studies. A gene gun device was used to introduce the recombinant plasmid into mice and the immune response was evaluated by ELISA, immunohistochemistry and immunoblot. The results showed that the chimeric CatL-Tm/Cyt cassette induced a high titer of $IgG_1$ and significant titers of $IgG_{2a}$ and $IgG_{2b}$ specific to CatL. This indicates a mixed Th1/Th2 response and demonstrates that pcDNA3.1-CatL-Tm/Cyt can be delivered as a functional DNA vaccine using immunization through a gene gun device.

**Keywords:** *Fasciola gigantica*; DNA vaccine; Cathepsin L; LAMP-1; Gene Gun

## 1. Introduction

DNA-based vaccines are constructed from genetic material encoding specific proteins or peptides. Previous studies have shown that DNA vaccines can induce significant immune responses against several infectious diseases [1]. DNA vaccines provide several advantages. They are easily designed and constructed through the use of genetic engineering [2,3], have no risk of reversion to a pathogenic form [4-6], and induce both humoral and cell-mediated immunity [7]. However, it has been shown that the immune response caused by DNA-based immunization is influenced by many factors such as the route of immunization, antigenicity of the encoded protein, co-stimulatory agents and intracellular mechanisms of the transfected cells. After experimental animals are immunized by a gene-gun device, then, the plasmid carrying the protein-encoding DNA insert must traverse the cell membrane, the cytoplasm and finally reach the nucleus. Here the

introduced coding sequence will be transcribed, driven by a strong constitutive promoter. The transcript will be transported to the cytoplasm and after translation the protein product will cause an immune responses via the Major Histocompatibility Complex (MHC) class I pathway [8,9]. MHC class I is the standard pathway for endogenous antigenic proteins, but it has also been observed that these antigens can be processed via the MHC class II pathway to induce CD4 T-cells [10]. This is the rationale of the presented work, to enhance the processing of endogenous antigenic proteins via the MHC class II pathway because clonal expansion, differentiation of peptide-specific B-cells and antibody production require cytokines for activation from CD4 T-cells.

Normally, CD4 T-cell activation occurs after endocytosis or phagocytosis of an exogenous antigenic protein, which is then degraded into small peptides in the endosomal or lysosomal compartments [11-14]. For this reason, we used the lysosomal associated membrane protein-1 (LAMP-1) in conjunction with CatL to facilitate presentation of endogenous synthesized protein on the cell membrane via MHC class II to T-cell receptors (TCR) of CD4 T-cells. LAMP-1 is localized in the membrane of lysosomal compartments [15]. The LAMP-1 trafficking pathway is located in the same place as the MHC class II trafficking pathway of immature Antigen Presenting Cells (APCs), and the formation of epitope or small peptide MHC class II complexes also takes place [16-20]. LAMP-1 if present on the cell-surface, can serve as a ligand for selectins [21,22], and also mediate cell-cell adhesion/recognition [23]. In this study a LAMP-1 DNA insertion cassette was constructed and functionally tested with a DNA fragment encoding CatL from *Fasciola gigantica*. This trematode parasite is responsible for huge economic losses in the meat industry [24]. A study by the WHO reported that 2.4 million people are infected with *Fasciola* and that 180 million people are at risk of infection [6]. In the tropics, fascioliasis has been reported as an important disease of cattle [25] with prevalence rates of 30-90% in Africa[26] and 85% in Northeast Thailand [27]. The aim of this research work is to increase the number of endogenous synthesized CatL proteins presented via the MHC class II pathway for CD4 T-cell activation and subsequent stimulation of B-cells for antibody production against CatL epitopes. CatL is the most abundant component of the adult parasite's excretory/secretory material and is also present in the juvenile parasite [28]. Therefore, this protein should be useful for protection against liver fluke infection.

## 2. Materials and methods

### 2.1 RNA isolation and first strand cDNA preparation

Total RNA of a mouse was extracted from liver tissue, which has a high level of LAMP-1 gene expression. The tissue was homogenized in 1 ml TRIzol reagent (GIBCO BRL Life Technologies, USA) by using a tissue homogenizer (IKA, Germany) and the RNA was then extracted following the supplied TRIzol protocol. The RNA was finally dissolved in DEPC-treated water and analyzed by agarose gel electrophoresis for its integrity. It was stored at −70°C until used for reverse transcription. First-strand cDNA was synthesized by using SuperScript™ II RT (Invitrogen, USA) and 1−5 μg of mouse liver RNA together with a (dT)12-18 oligonucleotide primer, according to the standard protocol provided with the enzyme.

### 2.2 Construction of pcDNA3.1-CatL-Tm/Cyt from mouse LAMP-1 cDNA and *F. gigantica* cathepsin L cDNA

The cDNA prepared as described above served as a template for PCR to amplify the mouse LAMP-1 Tm/Cyt fragment. *F. gigantica* CatL was amplified by

PCR using a previously cloned cDNA [29] inserted in pBluescript SK(-) (Startragene, USA)as a template. The following oligonucleotide primers were used $F_{cat}$ 5′-GAT CC GGTACC (*Kpn* I) ATG CGA TTG TTC ATA TTA GC-3′, $R_{cat}$ 5′-AAA A GGATCC (*Bam*H I) CGG AAA TCG TGC CAC CCA T-3′, $F_{Tm/Cyt}$ 5′-CCT CGA GGATCC (*Bam*H I) TTG ATC CCC ATT GCT GTG GGG C-3′, $R_{Tm/Cyt}$ 5′AAAACTCGAG (*Xho* I) CTA GAT GGT CTG ATA GCC GGC-3′. A standard PCR using *Taq* polymerase (Fermentas, Canada) was performed under the following conditions: Pre-denaturing at 94°C, 4 min for one cycle; followed by 30 cycles at 94°C, 1 min, 60°C, 1 min and 72°C, 1 min; and finally, 72°C for 10 min. The PCR products were analyzed by gel electrophoresis, purified and cloned into pGEM-T Easy (Promega, USA). After sequence verification, the cDNA fragments were isolated from pGEM-T Easy using the restriction enzyme recognition sites introduced by the PCR primers and finally subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen, USA) using the same restrictiton sites. The construct used for DNA immunization consisted of the coding sequence of *F. gigantica* CatL at its 5′-end combined with the mouse LAMP-1 transmembrane and cytoplasmic domains at its 3′-end. *E. coli* DH5α was transformed with the recombinant pcDNA3.1-CatL-Tm/Cyt DNA for the production of plasmid DNA to be used in later DNA-immunization experiments. Plasmid DNA was purified from cultures of transformant bacteria using the EndoFree Plasmid Maxi Kit (QIAGEN, USA) to eliminate endotoxin from *E. coli*.

### 2.3 Animals and Vaccination

Ten female Balb/C mice, 6−8 weeks old, were immunized with pcDNA3.1-CatL-Tm/Cyt DNA using the gold particle-mediated method via a gene gun device (Bio-Rad, USA) following the schedule shown in Table 1. In addition, immunization with unmodified pcDNA3.1 by the same schedule was performed as a negative control. The plasmid DNA was coated onto 1-mm gold particles (Bio-Rad, USA). Gold particles**(**25mg**)** were mixed with 100 µl 50 mM spermidine (Sigma, USA), 50 mg plasmid DNA, and 100 µl 1 M $CaCl_2$ (Sigma, USA). The mixture was precipitated at room temperature for 10 min and washed three times in absolute ethanol (Merck, Germany). The pellet of particles was resuspended in 3.5 ml 50 µg/ml polyvinylpyrrolidone (PVP, Bio-Rad, USA) in absolute ethanol. The suspension was loaded into Gold-Coat tubing and allowed to settle in a horizontal position for 3 min in a Tubing Prep Station (Bio-Rad, USA). Ethanol was removed and, while rotating, the tube was dried by a flow of nitrogen gas. The dried tubing was cut into 0.5 inch long pieces (cartridges) by a tube cutter (Bio-Rad, USA). The cartridges were stored in a tightly capped vial, containing a desiccant pellet, at 4°C. Each cartridge contained 1 µg of DNA coated onto 0.5 µg of gold particles. The DNA coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (Bio-Rad, USA) with a discharge pressure of 400 p.s.i.

### 2.4 Collection of immune sera

Mouse immune sera were obtained from the tail vein before the prime immunization and periodically at the days indicated in Table 1 after immunization.

### 2.5 Preparation of crude worm antigens

Crude worm (CW) extract was used as antigen to analyze the immune response in the DNA vaccination. Adult worms were suspended and homogenized in lysis buffer (10 mM Tris HCl, pH 7.2, 150 mM NaCl, 0.5% Triton-X 100, 1 mM EDTA, pH 7.2, 1 mM phenyl-methyl-sulphonyl-fluoride (PM SF)). The homogenate was rotated at 4°C for 1 h and insoluble material pelleted at 12000 rpm for 15 min. The supernatant was

collected and used as crude worm extract.

### 2.6 Analysis of humoral immune response by indirect ELISA

Standard 96-well plates were coated with 0.125 µg CW extract per well in 0.1 M sodium bicarbonate buffer, pH 9.6 at 4°C overnight. After three washes in PBS, 0.05% Tween 20 the plates were blocked with 0.25% BSA in PBS and incubated at 37°C for 30 min. The coated wells were filled with serum (see Table 2 for dilutions) in 0.25% BSA in PBS, incubated at 37°C for 2 h and washed as above. Ig-class/ subclass specific goat anti-mouse antibodies conjugated with horseradish peroxidase (see Table 2 for used Ig-class/subclass and dilution) in 0.25% BSA in PBS were added and the plates were incubated at 37°C for 1 h. Following washes as above the peroxidase substrate *Fast*™ OPD (Sigma, USA) was added. The reaction was stopped with 3 N $H_2SO_4$ and the absorption measured at 492 nm in an ELISA reader. Statistical comparison of the $A_{492}$ values recorded from vaccinated and non-vaccinated groups was performed using the non-parametric Friedman test in SPSS version 11.0.

### 2.7 Immunohistochemical staining of adult stage parasite tissue with immune sera

Tissue sections (8 µm thick) of juvenile *F. gigantica* (kindly provided by Poom Adisakwattana) were used for immunolocalization. The sections were prepared for incubation with immune sera as previously described [30] and were then incubated in sera (dilution) of mice immunized with pcDNA3.1-CatL-Tm/Cy or pcDNA3.1 in a humid chamber at 4°C overnight. Control sections were incubated with mouse preimmune sera (dilution). After three washes in PBS, 1% Tween 20 for 5 min each the sections were incubated with HRP-conjugated streptavidin (1:200) at room temperature for 30 min. The sections were washed as above and then washed again in TBS, pH 7.6 for 5 min. ExtrAvidin peroxidase was prepared following the manufacturer's protocol (ABcomplex/HRP, Dako Cytomation, Denmark), added to the sections and the sections were then incubated in a humid chamber at room temperature for 30 min. Following washes as above the sections were covered with substrate solution containing AEC-red (Zymed, USA). The reaction was stopped by rinsing with DDW and the sections were mounted in glycerol, PBS buffer at a ratio of 1:9 and observed and photographed under a light microscope.

### 2.8 SDS-PAGE and immunoblotting

CW extract of *F. gigantica* was size separated by 12.5% SDS-PAGE [31] in the Hoefer system (Amersham Biosciences, USA). After electrophoresis, proteins were stained with Coomassie blue to verify integrity or transferred to a nitrocellulose membrane (Biometra, Germany). The membrane was cut in strips, which were blocked with 5% skim milk in PBS for 2 h and then washed three times with PBS, 0.05% Tween 20. The strips were probed with individual diluted mouse sera (1:100) for 2 h. A HRP-Goat anti mouse Ig (H+L) (Southern Biotech) diluted 1:1000 was used as the secondary antibody and the reaction was developed with SIGMA*FAST*™ 3,3′-diaminobenzidine tetrahydrochloride with metal enhancer (DAB) (Sigma, USA) as chromogenic substrate.

## 3. Results

A 144 bp LAMP-1 cDNA fragment encoding the protein's transmembrane/ cytoplasmic domain was cloned by RT-PCR from mouse liver RNA and a 981 bp *F. gigantica* CatL cDNA containing the full coding sequence was amplified from pBluescript SK(−)-CatL-A (Fig. 1A, B). The CatL cDNA was inserted 5′ to the LAMP-1 Tm/Cyt cDNA into the pcDNA3.1

mammalian expression vector and the recombinant plasmid (pcDNA3.1-CatL-Tm/Cyt, Figure 1C) was then introduced into *E. coli* by transformation. Plasmid DNA of randomly selected transformant bacterial clones was tested by restriction enzyme analysis for the correct insert size and the sequence of the full CatL-Tm/Cyt construct was subsequently verified by DNA sequencing.

*F. gigantica* CW extract was used as an antigen in indirect ELISA to analyze the immune response of the mice during and after the immunization protocol. Sera of untreated mice served as a second negative control in addition to sera of mice immunized with the unmodified pcDNA3.1 vector. Mice immunized with pcDNA3.1-CatL-Tm/Cyt had significantly ($p<0.001$) higher $A_{492}$ values for $IgG_1$ compared to the control groups starting from the 1st boost (Day 28) and the values remained high until 5 weeks after the 2nd Boost (Day 77, Table 3). The $IgG_{2a}$ and $IgG_{2b}$ subclasses had also significantly ($p<0.05$) higher $A_{492}$ values compared to the control groups starting from the 2nd boost (Day 42) until 5 weeks after the 2nd Boost (Day 77, Table 3). IgA and IgM values did not differ from the control groups during the observation time (Table 3). The sera were also tested for antigen-specificity by using them for immunohistochemical staining of tissue sections prepared from juvenile parasites. Specific staining was observed in the parasite's intestinal epithelium with sera from pcDNA3.1-CatL-Tm/Cyt immunized mice while sera from pcDNA3.1 immunized mice and untreated mice were negative (Fig. 2). In immunoblotted CW extract proteins of the expected molecular weights, ~36 kDa for pro-cathepsin L and ~28 kDa for the mature enzyme, were reactive with sera from pcDNA3.1-CatL-Tm/Cyt immunized mice but not with sera from pcDNA3.1 immunized mice or untreated control mice (Fig. 3).

## 4. Discussion and conclusions

Previous studies demonstrated that cattle, naturally infected with *F. hepatica*, generate a predominant $IgG_1$ response and show only a low $IgG_2$ antibody titer [32,33]. Also it was found that the intraperitoneal injection of liver fluke excretory/secretory product can induce Th2 responses in Balb/C mice similar to those observed during infection [34]. This suggests that the immune response to liver fluke antigens is Th2 dominant. The results of our study demonstrate that pcDNA3.1-CatL-Tm/Cyt can induce a dominant $IgG_1$ response and, in addition, generate significant amounts of $IgG_{2a}$, $IgG_{2b}$. It indicates that administration of pcDNA3.1-CatL-Tm/Cyt DNA through a gene gun device induces a mixed Th1/Th2 response with a highly dominant Th2 response. However, this data needs to be confirmed by measurement of the cytokine response. A construct containing only CatL in pcDNA3.1 was unfortunately not stable in repeated cloning attempts. The bacteria (*E. coli* XL-1 blue) partially deleted the insert during culture. Therefore, this report cannot clearly explain whether the observed type of immune response is due to the LAMP-1 Tm/Cyt sequence, the CatL sequence or a combination of both. The plasmid alone did not cause an immune response in the control immunization. Specificity of the response was demonstrated by immunohistochemistry which only showed staining in the parasite's gut epithelium with sera from pcDNA3.1-CatL-Tm/Cyt immunized mice. Cathepsin L of *F. gigantica* had previously been demonstrated to be a gut-specific protein [29]. Also in immunoblots of CW extracts, cathepsin L was only detected by the sera of pcDNA3.1-CatL-Tm/Cyt immunized mice. Slightly differently sized bands are due to the presence of several CatL isoforms in the parasite.

In conclusion, we have demonstrated that DNA-based immunization using

pcDNA3.1-CatL-Tm/Cyt elicits a humoral immune response, predominant in $IgG_1$ but also with significant amounts of $IgG_{2a}$ and $IgG_{2b}$, specific to *F. gigantica* cathepsin L. Further studies are necessary to evaluate the function of LAMP-1 Tm/Cyt in this vaccine and the protective efficacy of this vaccine in the experimental mouse model.

## 5. Acknowledgements

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## 6. References

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**Table 1** Time schedule for DNA immunization

| Day: | 0 | 7 | 14 | 21 | 28 | 35 | 42 | 49 | 56 | 63 | 70 | 77 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bleeding | × | | × | | × | | × | | | | | × |
| DNA immunization | | × | | × | | × | | × | | | | |

**Table 2** List of anti-Ig antibodies used for analysis of immune response

| Ig Type | Serum dilution | Conjugate dilution |
|---|---|---|
| $IgG_1$ | 1:100 | 1:1000 |
| $IgG_{2a}$ | 1:100 | 1:1000 |
| $IgG_{2b}$ | 1:100 | 1:1000 |
| IgA | 1:50 | 1:500 |
| IgM | 1:100 | 1:1000 |

**Table 3** Measurement ($A_{492}$ ± standard deviation) of humoral immune response by indirect ELISA

| Group (N = 10)/Ig | Day 0 | Day 14 | Day 28 | Day 42 | Day 77 |
|---|---|---|---|---|---|
| **$IgG_1$** | | | | | |
| Untreated | 0.177±0.313 | 0.194±0.371 | 0.226±0.440 | 0.340±0.586 | 0.623±0.808 |
| pcDNA3.1 | 0.150±0.195 | 0.140±0.132 | 0.239±0.208 | 0.425±0.351 | 0.483±0.479 |
| pcDNA3.1-CatL-Tm/Cyt | 0.234±0.423 | 0.437±0.395 | 1.222±0.581$^{\#}$ | 2.800±0.374$^{\#}$ | 2.623±0.396$^{\#}$ |
| **$IgG_{2a}$** | | | | | |
| Untreated | 0.346±0.110 | 0.204±0.129 | 0.372±0.165 | 0.361±0.210 | 0.352±0.129 |
| pcDNA3.1 | 0.303±0.151 | 0.277±0.138 | 0.284±0.069 | 0.424±0.153 | 0.320±0.139 |

**Table 3** Measurement ($A_{492}$ ± standard deviation) of humoral immune response by indirect ELISA (Cont.)

| Group (N = 10)/Ig | Day 0 | Day 14 | Day 28 | Day 42 | Day 77 |
|---|---|---|---|---|---|
| pcDNA3.1-CatL-Tm/Cyt | 0.339±0.142 | 0.285±0.223 | 0.472±0.201 | 0.706±0.175* | 0.688±0.273* |
| **$IgG_{2b}$** | | | | | |
| Untreated | 0.228±0.111 | 0.346±0.126 | 0.262±0.105 | 0.218±0.088 | 0.162±0.106 |
| pcDNA3.1 | 0.314±0.126 | 0.303±0.131 | 0.382±0.217 | 0.152±0.144 | 0.237±0.181 |
| pcDNA3.1-CatL-Tm/Cyt | 0.206±0.146 | 0.322±0.140 | 0.357±0.129 | 0.779±0.400# | 0.548±0.323* |
| **IgA** | | | | | |
| Untreated | 0.193±0.116 | 0.179±0.137 | 0.085±0.084 | 0.150±0.106 | 0.143±0.132 |
| pcDNA3.1 | 0.096±0.112 | 0.292±0.329 | 0.092±0.100 | 0.167±0.116 | 0.295±0.166 |
| pcDNA3.1-CatL-Tm/Cyt | 0.148±0.098 | 0.164±0.119 | 0.126±0.165 | 0.112±0.093 | 0.314±0.188 |
| **IgM** | | | | | |
| Untreated | 0.201±0.149 | 0.146±0.137 | 0.403±0.482 | 0.314±0.158 | 0.325±0.161 |
| pcDNA3.1 | 0.393±0.234 | 0.304±0.163 | 0.322±0.141 | 0.394±0.143 | 0.408±0.183 |
| pcDNA3.1-CatL-Tm/Cyt | 0.289±0.155 | 0.314±0.188 | 0.316±0.125 | 0.471±0.184 | 0.402±0.170 |

Significant different to controls with # ($p<0.001$) and * ($p<0.05$).

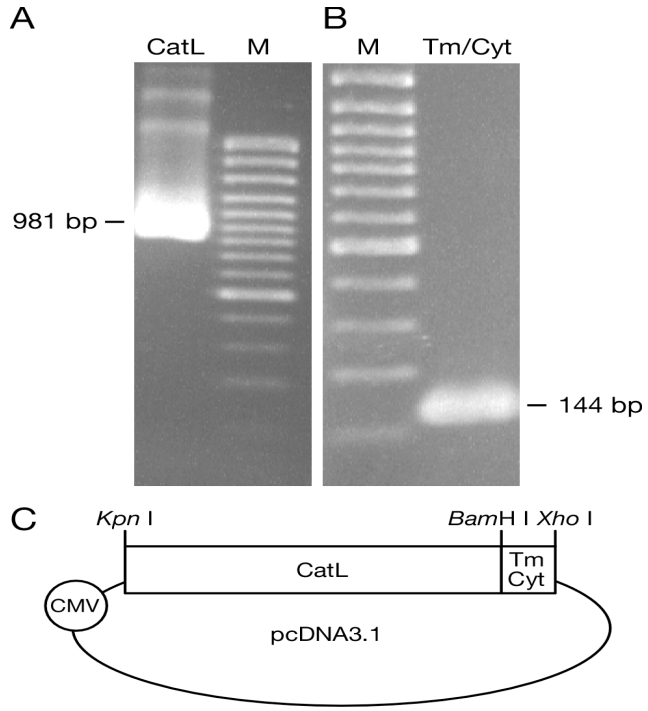


**Fig. 1** Agarose gel (1%) electrophoresis of PCR products and schematic drawing of the CatL-Tm/Cyt cassette in the expression vector pcDNA3.1. **A**, *F. gigantica* 981 bp CatL cDNA; **B**, *M. musculus* 144 bp LAMP-1 Tm/Cyt cDNA. M, 100 bp ladder. **C**, schematic drawing of the CatL-Tm/Cyt cassette in the expression vector pcDNA3.1. Indicated are the 981 bp cathepsin L cDNA, the 144 bp LAMP-1 transmembrane and cytoplasmic domain cDNA, the restriction sites *Kpn* I, *Bam*H I, *Xho* I used for insertion in the plasmid and the constitutive CMV

promoter.

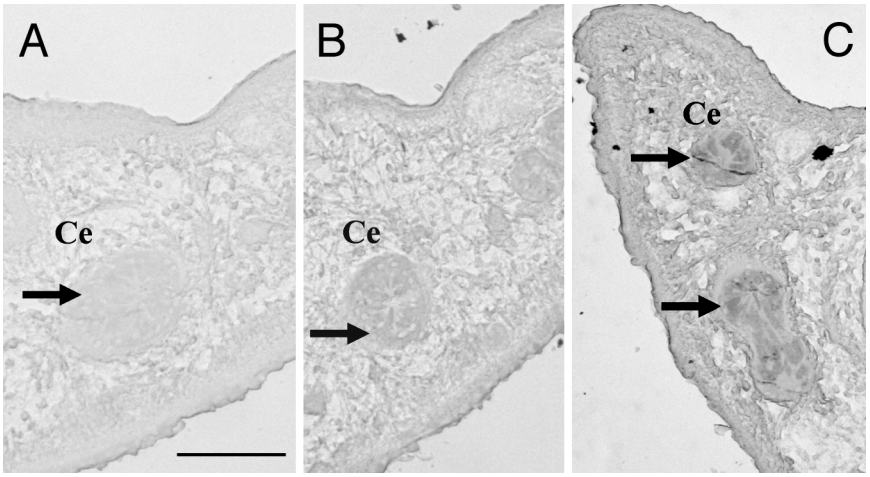


**Fig. 2** Immunohistochemical detection of cathepsin L in juvenile parasites. **A**, tissue probed with serum of a non-immunized mouse; **B**, tissue probed with serum of a pcDNA3.1 immunized mouse; **C**, tissue probed with serum of a pcDNA3.1-CatL-Tm/Cyt immunized mouse. Arrows point to ceca (Ce), scale bar: 100 µm.

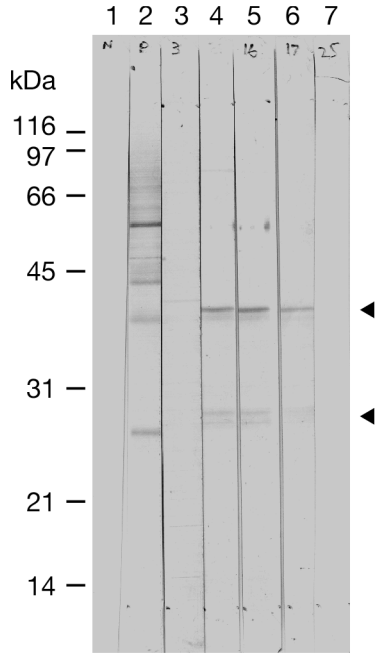


**Fig. 3** Detection of cathepsin L in immunoblotted CW extract of adult parasites. Lane 1: probed with pre-immune mouse serum (negative control), lane 2: probed with mouse infected serum (positive control), lane 3: probed with pcDNA3.1 immunized mouse serum, lanes 4-6: probed with pcDNA3.1-CatL-Tm/Cyt immunized mouse sera, lane 7: probed with non-immunized mouse serum. The positions of reactive pro-cathepsin L (~36 kDa) and mature cathepsin L (~28 kDa) isoforms are indicated by arrowheads. Positions and molecular weights of the broad range marker proteins (Bio-Rad) are indicated at the left side.