

## Research Article



## Design the New Method for Fasciolosis Detection Based on Quantum Dot Nanoparticle

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### ABSTRACT

The Fasciolatrematodes are a causative agent of liver fluke disease, fascioliasis, in mammals and have a world-wide distribution. Losses recent reports indicated that Fasciola is also a major human pathogen in Andean countries, South America, Northern Africa, areas in Western Europe as well as Iran and some other Asian countries. It is estimated that 2.4 million people are infected throughout the world. Hence detection of pathogenic subject of fasciola has been an important issue because of its critical impact on public health. Also, studies have shown that the most predominant molecules secreted by Fasciola hepatica parasites are cathepsin L (catL). The catL is the cysteine proteinase that plays major role in the lysosomal proteolysis in Fasciola hepatica penetration process. Present study described developing a nano biosensor based on quantum dots (QDs) for detection of fasciolosis in infected animals. The catL type one (catL1) was elected as a protein target for construction of specific binding antibody. The polyclonal antibodies (anti-catL1) were constructed and conjugated to Tioglycolic acid-modified Cadmium-Telluride QDs (CdTe-QDs) via electrostatic interaction. On the other hand, the dye (Rhodamine) molecules were attached to the catL1. In fact, the donor-acceptor complexes were formed based on the antigen antibody interaction (QDs-Ab-Ag-catL1).

**Keywords:** Fasciolatrematodes; quantum dots; cathepsin L; Rhodamine.

### INTRODUCTION

Fasciolosis is a disease caused by liver flukes of the genus fasciola. In cattle and sheep, Fasciola hepatica (temperate) and Fasciolagigantica (tropical) are the causative agents of liver fluke disease, fasciolosis. Fasciolosis is also an important food-borne zoonotic disease in humans and a common disease associated with domestic livestock and humans. Recently World Health Organization (WHO) was recognized fasciolosis as a serious human's public health problem. Furthermore, 2.4–17 million people and 91.1 million people are infected with fasciola and at risk of fasciola infection, respectively. Human liver flukes disease has global prevalence rate particularly in the Andean countries of South America, Egypt, Peru, Bolivia, Ecuador, Iran, and Vietnam where farming practices allow infected animals to roam among plants used for consumption. Also, fasciolosis causes world-wide economic losses of approximately US\$2,000 million annually to the agricultural sector<sup>1</sup>. Several outbreaks of this disease recently occurred in the Guilan province of Northern Iran and in 1999 alone over 10,000 individuals were infected.<sup>2-6</sup>

Fasciolosis is acquired following the ingestion of vegetation or water contaminated with the encysted infectious liver fluke larvae, known as metacercariae. Encysted metacercariae in the intestines, burrow through the intestinal wall and migrate into the liver tissue where they spend about 8–12 weeks feeding on host tissue and consequently causing extensive hemorrhaging and perforations. Immunological damages and tissue injuries were revealed before Fasciola move into the bile ducts.

The parasites can remain for up to 1–2 years in the bile ducts of cattle and as long as 20 years in sheep. This acute stage of disease can result in death in highly infected sheep, but death is rarely seen in cattle. After this period the parasites move into the bile ducts where they complete their growth and maturation steps. The hermaphroditic adult liver flukes puncture the wall of the bile duct and feed on blood that provides the nutrient for the production of enormous numbers of eggs that are carried into the intestine with the bile fluids and are passed onto pasture with the feces. An aquatic larval stage hatches from the eggs and infects an intermediate mud-snail host, such as *Lymnaea truncatula*. After several developmental and multiplication stages within the snail the parasites emerge and become encysted on vegetation and thus continue the cycle<sup>7</sup>. A number of chemicals are commercially available for the treatment of animal fasciolosis, including closantal, clorsulon, rafoxanide, nitroxynil and triclabendazole.<sup>8,9</sup>

Studies have shown that the most predominant molecules secreted by *F. hepatica* parasites in vitro are cathepsin L cysteine proteases that facilitate the penetration of the parasite through the tissues of host, and also participate in functions such as feeding and immune evasion. The secretion of proteases facilitates migration of the parasite through host tissue and the degradation of host macromolecules to provide essential free amino acids for the parasite. Furthermore, cathepsin L proteases are considered the principle participants; the parasite enzymes cleave host antibodies specifically in the hinge region to prevent antibody-mediated cell damage



and alter the function of cells of the innate and adaptive cellular immune systems to suppress the development of protective Th1-driven responses.<sup>10,11</sup>

Detection of pathogenic subject of fasciola has been an important issue for scientists because of its critical impact on public health. Although standard Immunological methods of detection are confirmative to identify fascioliasis, it often takes much time till several days to complete the processes such as Complement Fixation (CF), Immune Fluorescent Method (IFA), Counter Electrophoresis (CEP), Indirect Agglutination (IHA), Western Blot and ELISA (Fas2-ELISA and Ctl1-ELISA). In addition, most of conventional methods require intricate instrumentation and cannot be used on-site. Thus, both private and government sectors strongly need biosensors that can detect fascioliasis in a fast and accurate manner. The advances in the development of nanomaterials have stimulated worldwide research interests in their applications for bio analysis.

The incorporation of these functionalized nanomaterials into current pathogen detection methods has led to the development of new generation methods with emphasis on device portability and simplicity in sample preparation, improved sensitivity, rapid and nearly real-time (as short as a few minutes) detection. The conjugation of biomolecules with nanomaterials is the foundation of nano-biorecognition. A variety of strategies such as antibody–antigen recognitions have been explored for specific detection between target and bio-functionalized nanomaterials. Each nanoparticle (NP) with diameter of around 100 nm could efficiently conjugate about 150–200 molecules of antibody and result in more than 300 active binding sites (two binding sites for each antibody). Coating biomolecules on NPs allows multiple contacts between nanomaterials and target cells, and therefore, the functionalized nanomaterials display higher binding affinity than free biomolecules demonstrated that the binding affinity constant for antibody– NP bioconjugates was eightfold higher than the intrinsic affinity of the free antibody. One of the most highlighted and fastest moving interfaces of nanotechnology is the application of quantum dots (QDs) in biology. The unparalleled advantages of the size tunable fluorescent emission and the simultaneous excitation at a single wavelength make QDs the great possibility for use in optical encoding detection.<sup>12</sup>

In this work, donor–acceptor complexes were formed based on antibody–antigen interactions. Immunoglobulin (rabbit-IgG) was effectively conjugated to Tioglycolic acid modified CdTeQD synthesized in aqueous solution via covalent interaction, while organic dyes, fluorophores were attached to the cathepsinL1 (antigen). The mutual affinity of the antigen and antibody brought the CdTeQDs and Rhodamine sufficiently close together to allow the resonance dipole–dipole coupling required for fluorescence resonance energy transfer to occur. The formation of immune-complexes resulted in fluorescence

resonance energy transfer from the CdTeQDs donors to the Rhodamin acceptors. The assay was based on the detection of serum antibodies reactive with antigens secreted by the parasite and purified in laboratory to optimizing interaction.

## MATERIALS AND METHODS

### Parasite

Livers of infected sheep with Fasciola were obtained from a local abattoir at Guilan, Iran. Live intact *F. gigantica* adult worms were collected from the bile ducts and thoroughly washed at room temperature with several times with PBS[pH=7.4] and were incubated in the same buffer at 37°C for 3h to eliminate any residual host matter. After washing it stored at -70°C until used.

### Preparation of Total RNA

The total RNA was extracted using QIAGEN(R) RNA extraction kit(Q IAGEN(R), Germany). According to the manufacturer's instructions, total RNA was quantified by measuring the optical density at 260 nm and had qualitative by agarose gel electrophoresis using ethidium bromide for staining<sup>13</sup>.

### Expression and purification of recombinant protein

The gene encoding pro-cysteine proteinase (immature segment) of *F.gigantica* was amplified by PCR from cDNA using oligonucleotide primers with BamHI and EcoRI restriction enzyme sites added. The primers were designed from the nucleotide sequences which were determined in "Sequencing of cysteine proteinase of *F.gigantica*" as follows: FOR Fas1 (5'-AAAGGATCCATGAGATTGTTTCATATTAG -3'), REV Fas (5'-AAACTCGACTCACGGAAATCGTGCCAC -3') were based on the *F. gigantica* thepsin L1 sequence. XhoI and BamHI restriction sites were included for cloning purposes. PCR was performed and then purified PCR products were digested with BamHI and XhoI and were cloned into the pET28a<sup>+</sup> expression vector (Invitrogen).

The recombinant plasmid was transformed into an *Escherichia coli* BL21 strain; Positive transformants were determined by PCR screening and the expression of polyhistidine-containing recombinant proteins was induced by adding isopropyl-β-d-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37°C for 6 h. The induced cells were harvested and disrupted by sonication in 20 mM Tris-HCl buffer (pH 8.0).

The recombinant protein expressed as inclusion bodies was solubilized completely with 6 M urea in 20 mM Tris-HCl buffer (pH 8.0) and then subjected to a His tag column for affinity purification of histidine-tagged proteins according to the manufacturer's instructions.

The recombinant protein was eluted with imidazole, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) and stained with Coomassie brilliant blue to assess their purity.



### Preparation of Immune Sera from Rabbits

2male 1.5-2 Kilogram rabbits prepared. One rabbit Immunized with purified cathepsin antigen And Freund's adjuvant but another Injected with adjuvant alone as a control. Whole blood was collected from each rabbit after 5 time injections and centrifuged at 4000 rpm at 4°C for 10 minutes and the obtained serum samples were stored at -80°C until use.

### Preparation of CdTe QDs

The CdTe QDs were prepared according to the reported method<sup>14</sup>. Briefly, TGA (tio glycolic acid) was used as a stabilizing agent, and the molar ratio of Cd<sup>2+</sup>: stabilizer: HTe was fixed at 1:2.4:0.5. The resulting mixture was then subjected to a reflux 2 h to control the growth of the CdTe QDs, and then CdTe particles with a certain size were obtained.<sup>15</sup> The water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was used to activate the QD-COOH surface chemistry prior to their incubation with antibody (carboxyl/EDC/Ab).<sup>16</sup>

### Linking CdTe QDs with antibody

As antibody has a strong affinity for the carboxylate group of the linker molecules, TGA bifunctional linker molecules (HOOC-CH<sub>2</sub>-SH) with carboxylate and thiol functional groups were used to facilitate the binding of CdTe QDs to antibody. The obtained QDs were hydrophobic and dispersed in organic solvent. Before linking the QDs with antibody, the QDs should be modified with TGA, which contains one mercapto group and one carboxylate group. In this work, the CdTe QDs were synthesized by aqueous colloid approach as previously described. The obtained CdTe QDs were capped with TGA. The mercapto group is conjugated with the Cd ion on the surface of CdTeQDs, while the carboxylate group will be ionized in the water to make the CdTe QDs water soluble. The obtained QDs were absorbed by the antibody due to the carboxylate group peak as the origin QDs.

### Antigen attaching to rhodamin

The Rhodamine-CL1 (Rh-CL1) saturated antiCL1 antibody column used in immune-sensor was prepared according to the protocol developed in this laboratory. Fluorophores exhibit specific quantum yields. Fluorescence quantum yield is a measure of the efficiency with which a fluorophore is able to convert absorbed light to emitted light. Higher quantum yields result in higher fluorescence intensities. Interaction between QD/antibody and CL1/Rhodamine: Week interactions of mutual affinity between Ab/Agin aqueous brought QD and Rhodamine near together to occur FRET mechanism.

### Antibody-based fluoroimmunosensors (FISs)

Fluorescence resonance energy transfer (FRET) occurs when the electronic excitation energy of a donor chromophore is transferred to an acceptor molecule nearby via a through-space dipole–dipole interaction between the donor–acceptor pair. The FRET process is

more efficient when there is an appreciable overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. The strong distance dependence of FRET efficiency has been widely exploited in studying the structure and dynamics of proteins and nucleic acids, in the detection and visualization of intermolecular associations and in the development of intermolecular binding assays.<sup>17</sup>

### RESULTS AND DISCUSSION

Done PCR using Fas1 as template and designed primer exhibit 981 bp bands on agarose gel.(Figure 1)

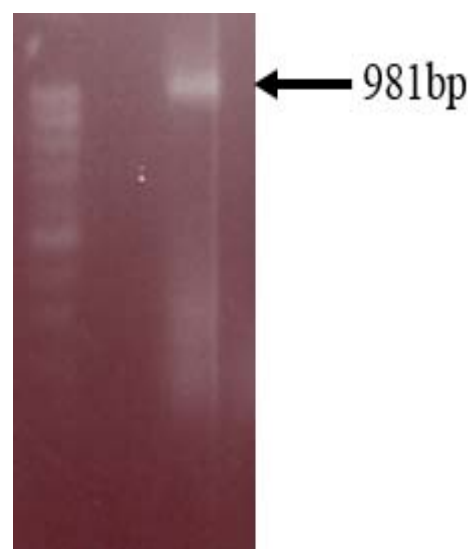


Figure 1: PCR from 981 bp

Isolation of positive colonies had done by colony-PCR and enzymatic digestion. (Figure 2)

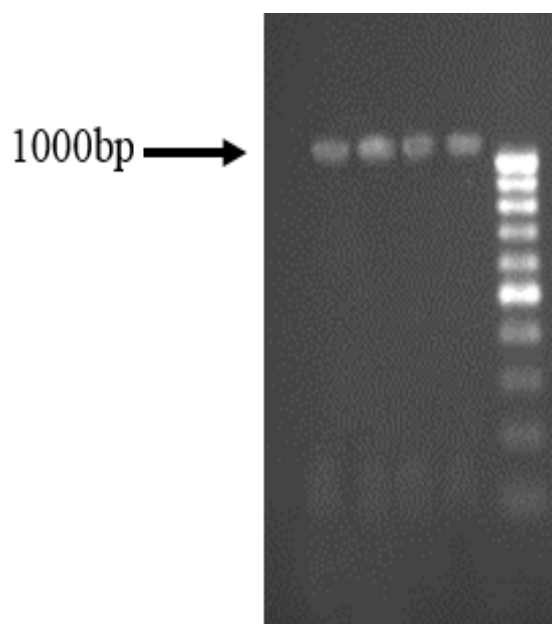


Figure 2: PCR cloning results

Target His-tagged fusion antigen were separated from culture by using Ni-agarose column and analyzed SDS-PAGE showed a single 37 kDa band indicating that the protein was purified (Figure 3 and 4).

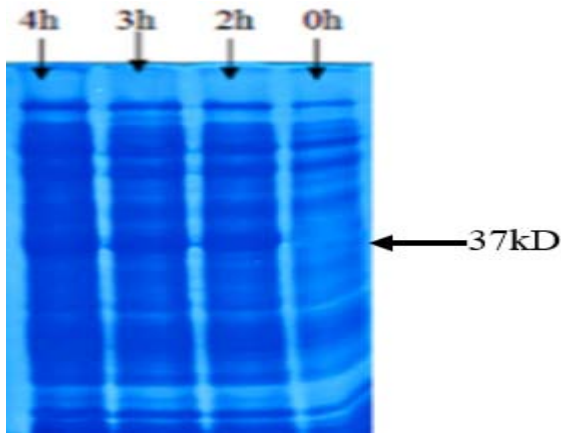


Figure 3: expression of Ctl1

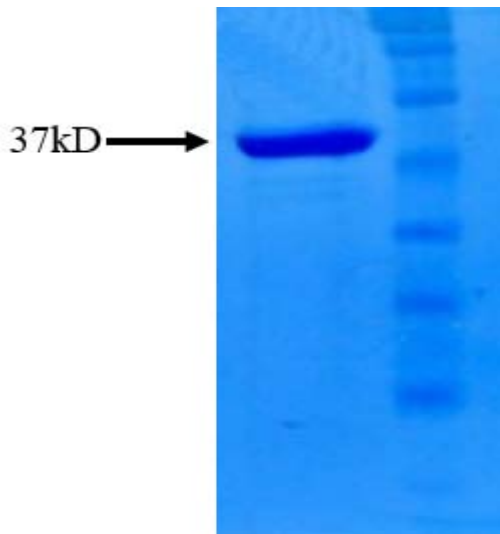


Figure 4: Ctl1 purification

Identification of the antigen concentration with Bradford method gives 266 µgr/ml. After bleeding from rabbit, titration of antibody with Immuno diffusion (gel diffusion) (Figure5), Dialysis & using DEAE sepharose chromatography for extract IgG has been done by Tris-HCL 100 mM pH=8.6 (Figure6).

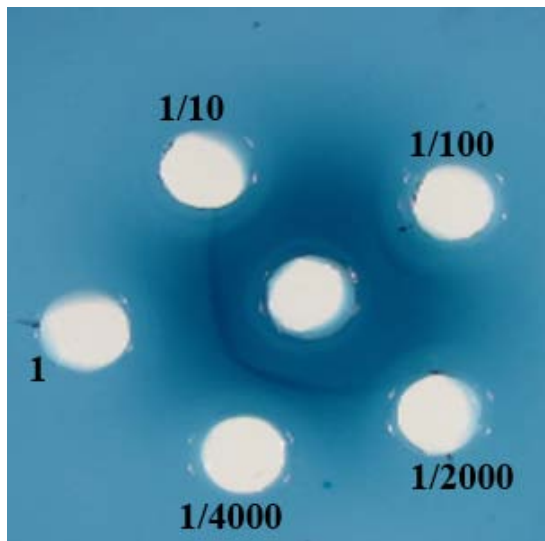


Figure 5: immunodiffusion

The IgG concentration was assayed with Bradford method.

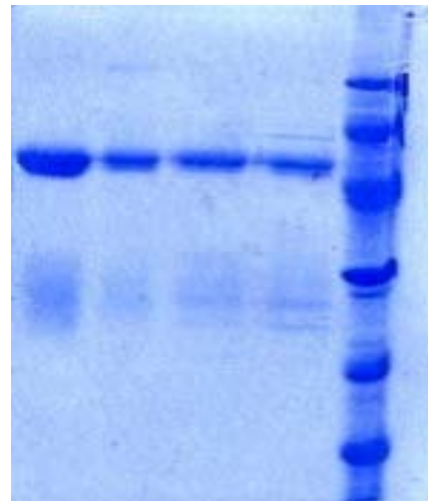
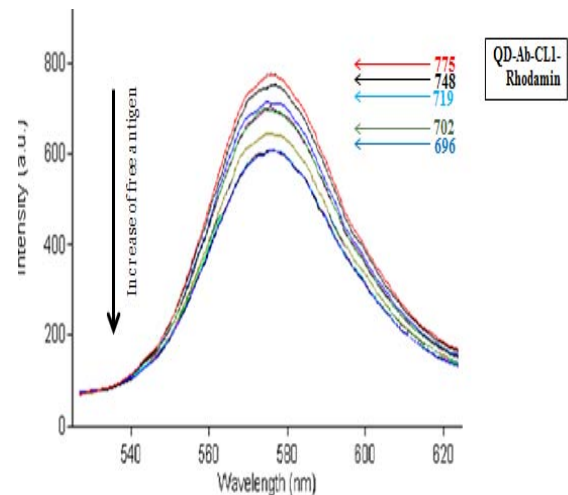


Figure 6: antibody purification

In this study, we explored the use of semiconductor QDs as fluorescence labels in immunoassays for simultaneous detection of Cathepsin L1, fasciolosis agent. Highly fluorescent semiconductor QDs with 460 nm emission wavelengths spectra were conjugated to anti-Ctl1 antibodies, respectively.

Fluoroimmunoassays using QD/antibody and Rhodamin/Ctl1 conjugates: Below Figure shows emission spectra obtained from CdTe QDs after being deposited into the antibody and interaction with antigen. The QDs exhibit characteristic emission peaks at 460 nm. After the QDs are binding to the antibody (QDs/Ab), the hybrid nanostructure exhibit same emission but when there are Ctl1/Rhodamine (labeled antigen) the interaction between antigen antibody and so FRET occur between Rhodamine and QDs. This prose transfers the emission spectra to 575. If free Ctl1 (unlabeled antigen) add to solution, the QD/Ab interaction with free antigen resulted to FRET off. (Depend on concentration of free antigen) and decrease in 575nm peak intensity, therefore observe an increasing in 460nmpeak intensity.





## CONCLUSION

Illnesses caused by food-borne pathogens, fascioliasis, have demonstrated the need for improved.

Estimated 180 million people in 51 country exposure at risk and 2.4 million people are infected every year.

This is the first report on designing a CL1-based biosensing tool for detection of fascioliasis in Iran. The techniques outlined in this report highlight some of the available tools, but as research continues the methods improve as well.

The advantages of this method are: (I) The immune-sensor constructed show a sensitivity with a detection limit of ng/ml, which could be used for real detection of fascioliasis, (II) Use antigen to early detection, (III) Detection of CatL1 without previous purification, (IV) Being simple and fast, (V) Very short time to detection of CatL1 (minutes), (VI) Stability of synthesized chemical component and (VII) reduced detection time.

As a nano-biosensor technology becomes more refined and reliable, it is likely it will eventually make its way from the lab to the clinic, where future lab-on-a-chip devices incorporating an array of nano-biosensors could be used for rapid screening of a wide variety of analytes at low cost using small samples of patient material.

In future we hope to approve this method such as: optimization this method for increasing sensitivity, Compare the sensitivity of this method with other detection methods and increasing the specificity of this method by using aptamers to decrease the cross reactivity with other similar enzymes.

Finally we hope to develop this method as an efficient, rapid and high sensitive test, however; it would be need more *in vivo* and *in vitro* studies.

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## REFERENCES

- Spithill TW; Smooker PM; Copeman DB (1999). "Fasciolagigantica: epidemiology, control, immunology and molecular biology". In Dalton, JP. Fasciolosis. Wallingford, Oxon, UK: CABI Pub. pp. 465–525. ISBN 0-85199-260-9.
- N. Amor, A. Halajian, P. Merella, S. Farjallah, K. Said, and B. Ben, "Genetic Characterization of Fasciolaspp. From Tonekabon City (Northern Iran) Based on the Ribosomal Internal Transcribed Spacer Regions," *City*, vol. 43, no. 6, 2011, 1061-1067.
- O. Article, "Nuclear Ribosomal DNA ITS-2 Sequence Characterization of Fasciola hepatica and Galba truncatula," *Iranian Journal of Public Health*, vol. 36, no. 4, 2007, 42-49.
- K. Ashrafi, J. Golchai, and S. Geranmayeh, "Human Subcutaneous Dirofilaria due to Dirofilaria (Nochtiella) repens: Clinically Suspected as Cutaneous Fascioliasis," *Iranian Journal of Public Health*, vol. 39, no. 1, 2010, 105-109.
- P. R. Collins et al. Cathepsin L1, the major protease involved in liver fluke (Fasciola hepatica) virulence: propetide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. *The Journal of biological chemistry*, vol. 279, no. 17, Apr. 2004, 17038-46.
- Mulcahy G, O'Connor F, Clery D, Hogan SF, Dowd AJ, Andrews SJ, Dalton JP, Immune responses of cattle to experimental anti-Fasciola hepatica vaccines, *Res Vet Sci*. 67(1), Aug 1999, 27-33.
- J. P. Dalton et al., "Fasciola hepatica cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines," *International Journal for Parasitology*, vol. 33, no. 11, Sep. 2003, 1173-1181.
- D. J. Dowling et al., "Major secretory antigens of the helminth Fasciola hepatica activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses.," *Infection and immunity*, vol. 78, no. 2, Mar. 2010, 793-801.
- K. Ishidoh and E. Kominami, "Multi-step processing of procathepsin L in vitro.," *FEBS letters*, vol. 352, no. 3, Oct. 1994, 281-4.
- S. Kuk, M. Kaplan, A. Ozdarendeli, S. Tonbak, S. Felek, and A. Kalkan, "Fasciola hepatica cathepsin L1 from a Turkish isolate is related to Asiatic isolates," *ActaParasitologica* vol. 50, no. 3, 2005, 244-248.
- L. Marcos, V. Maco, A. Terashima, F. Samalvides, J. R. Espinoza, and E. Gotuzzo, Fascioliasis in relatives of patients with Fasciola hepatica infection in Peru., *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 47, no. 4, 2005, 219-22.
- R. W. Mason, Species variants of cathepsin L and their immunological identification., *The Biochemical journal*, vol. 240, no. 1, Nov. 1986, 285-8.
- Protein folding in the cell, Mary-Jane Gething and Joseph Sambrook Department of Biochemistry, University of Texas Southwestern Medical Centre, 5323 Harry Mines Boulevard, Dallas, Texas 75235, USA. *Nature*, 355, January 1992, 33-45.
- Hao Zhang, Zhen Zhou and Bai Yang Key, The Influence of Carboxyl Groups on the Photoluminescence of Mercaptocarboxylic Acid-Stabilized CdTe Nanoparticles. *J. Phys. Chem. B*, 107(1), 2003, 8–13.
- M. Cordova, L. Reategui, J.R. Espinoza. Immunodiagnosis of human fascioliasis with Fasciola



- hepatica cysteine proteinases. *Trans. R. Soc. Trop. Med. Hyg.* 93, 1999, 54–57.
16. J. Parasitol and O. Article, *Strongyloidesstercoralis*: The Most Prevalent Parasitic Cause of Eosinophilia in Gilan Province, Northern Iran, *Parasitology*, vol. 5, no. 3, 2010, 40-47.
17. S. M. O'Neill, M. Parkinson, a J. Dowd, W. Strauss, R. Angles, and J. P. Dalton. Short report: Immunodiagnosis of human fascioliasis using recombinant *Fasciola hepatica* cathepsin L1 cysteine proteinase. *The American journal of tropical medicine and hygiene*, vol. 60, no. 5, May. 1999, 749-51.

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