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Human Hydatid Cyst Zoonotic Characters and analysis of P29 Recombinant Protein Immunogenicity

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ABSTRACT

Introduction: Echinococcosis or hydatid disease is one of the most important zoonosis across the world.
Materials and Methods: The recombinant P29 protein is considered as a suitable candidate for evaluating diagnostic ELISA to reach a more accurate conclusion.
Results: In this study the P29 sequence comprised with somewhat similar sequences registered in Gene Bank and showed 100% identity with P29 sequences derived from *Echinococcus granolosus* (*E. granulosus*) (XP_024351425.1), *E. multilocularis* (AHA85396.1), Endophilin B1 protein of *E. granulosus* (CDS21096.1). The primary and secondary structure of the peptide was analyzed due to its characterization of peptide motifs to be expressed on MHC Class HLA allels.
Conclusion: The recombinant P29 derived peptides can be produced and analyzed due to be determined as vaccine design candidate against Hydatidosis.
Keywords: *Echinococcus granolosus*, Hydatidosis, MHC, P29, Recombinant Antigen

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Introduction

Echinococcosis or hydatid disease is one of the most important zoonosis across the world. The causative agent is the metacestode stage of *Echinococcus granolosus* (*E. granulosus*) that infects humans and many other mammals as its intermediate hosts (1). Several diagnostic imaging tools has been used in order to identify infection, (2) but the necessity of rapid reliable screening tests for early diagnosis of new cystic echinococcosis (CE) and follow-up treated CE cases, led to design and production of novel serological tests such as produce recombinant proteins and species-specific antigens for detection the CE infection (3). There are many antigens in fluid taken from the cyst, however the complex mixture of its substances and the immunological cross reactions led to design recombinant Ags

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taken from different parts of the parasite (4). Two-D Clean-up Kit and dialysis of three-D images of separated spots were done and the use of Progenesis SameSpots software showed the best result (5). The relationship between the parasite and the host can be handled by several intermediate protein such as P29 antigen. Since the antigen B known as the main diagnostic Ag for the disease has the cross reaction with P29, it can be considered as a diagnostic tool or vaccine preparation process (6). P29 is not secreted outside the infected cells and is expressed in all stages of the parasite life cycle, therefore can play a major role in providing immunity against the parasite. The P29 antigen has been considered as a good candidate for monitoring of the patients after the surgical process of Hydatid cyst (7). A recent study confirmed that in mice immunized with rEg.P29 the activation of T cells will be elicited and amino acids 86-100 of rEg.P29 as T cell receptor epitopes elicit significant antigen-specific IFN-γ production in CD4 and CD8 T cells and promotes specific T-cell activation and proliferation (8). REgP29 could also be considered as an effective DNA vaccine for controlling of *E. granulosus* prevalence in intermediated host (9). Class I molecules of the MHC Cytotoxic T lymphocytes (CTLs) express peptides derived from parasitic and viral infections as a part of the immune system concerned with recognition of host cells that express new antigens (10). The structure of HLA-B27 shows nonamer self-peptides bound in an extended conformation (11). Newly synthesized proteins are degraded into peptides in the cytoplasm, and after be transported to the endoplasmic reticulum will be bound to class I molecules, and then expressed on the cell surface (12). All the process is controlled by a gene in the human major histocompatibility complex class II region (13).

MHC Major histocompatibility complex class II (MHC II) molecules are expressed on the surface of antigen-presenting cells and initiate the CD4(+) T cell response (14). In this study the character of recombinant P29 to be expressed on MHC molecules was evaluated.

Materials and Methods

Gene synthesis was carried out by the ShineGene Company (Shanghai, China) in length of P29 as 730 bp. This gene in the pUCc57 public vector was sent by the manufacturer. Expression plasmid pET-28a, carrying the resistance gene to Kanamycin, was considered as the vector for the P29 gene. The cut p29 gene was cloned by ligation of the T4DNA ligase enzyme in the pET-28a vector and was transformed into *E. coli* DH5a bacterium. Then, the bacteria containing the recombinant vector were cultivated on agar plates. Finally, the recombinant plasmid was introduced into Escherichia coli strain BL21, and recombinant plasmid P29/pET-28a was cultured on kanamycin plat. Separation of the recombinant protein was carried out by the kit based on the isolation of histidine-sequenced proteins (15). The recombinant P29 synthesized gene was about 730bp. The recombinant DNA was first sequenced and comprised to other P29 sequences registered in GeneBank under accession numbers XP_024351425.1, AHA85396.1, CDS21096.1 and CAE1271104.1. The latest accession number was determined as a far species with low homology percentage rate. Sequences were aligned using BioEdit ClustalW multiple Allignment program. The insert DNA was then cloned in pET-28a vector and expressed in *E. coli* strain BL21. All the results are published and discussed previously (15) and the specific recombinant P29 protein is discussed here. 2nd and 3rd structures of P29 peptide were predicted and analysed using **ProtParam** online tool and the availability of P29 different motifs to be expressed on MHC class I and II HLA-alleles were predicted using Genscript online tool. For analysis of immunoblotting, after separation of proteins on the SDS-PAGE gel, using the

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western blotting method, they were transferred to the nitrocellulose membrane. After the electrophoresis stage, the nitrocellulose membrane was cut to gel size and was placed in the transfer buffer (including methanol 20%, Tris base 23 mM, and 192 mM Glycine) for 10 min. Then, the gel and membrane were placed inside the Bio-Rad western transfer system, according to the protocol. The transformation from gel to the membrane was done at the voltage of 100v, the temperature of 4 ºC for 60 min. Then, the membrane was placed in the blocking buffer containing BSA 5% and NaCl 137 mM, and Na2HPO4 4.3 mM and KCl 2.7 mM at 4ºC overnight. Then, the membrane was washed in three steps with phosphate buffer containing Tween 20 0.05% (10-min shaking for each time). After washing the membrane, AntiHis monoclonal antibody was added to the membrane at an initial dilution ratio of 1:5000, and the membrane was placed on a shaker for 90 min at room temperature in a buffer. again, and the membrane was washed with PBST buffer (including NaCl 137 mM and Na2HPO4 4.3 mM, KCl 2.7 mM and KH2PO4 1.4 mM, and Tween20 0.05%) in 3 10-min steps. Then, the membrane was styled in a buffer containing HRP conjugated anti-His Antibody gout with a dilution ratio of 1:8000 as a secondary antibody for 90 min. The membrane was again washed in a 10-min buffer. In the end, -diaminobenzidine (DAB) 3.3 at 25 mg of concentration was dissolved in 50 ml of Tris 100 mm buffer, and the membrane was placed in it. After the bands were determined, the coloring reaction was stopped by washing with distilled water (15). 25 human serum samples obtained from infected people who undergo to hydatid cyst surgery in Emam Khomeini Hospital, related to Tehran University of Medical Sciences were collected. Human serums with 5 different dilute of 1:10, 1:100, 1:200, 1:400, and 1:800 were analyzed based on indirect ELISA method using the prepared P29 recombinant antigen. Also, 20 negative control serums, from healthy individuals without hydatidosis infection were utilized in the ELISA, mainly adolescents and children. For verification of tests, all subjects also were evaluated using an ELISA commercial kit (Pishtaz Medicine, Tehran, Iran). All samples were stored at -30 ºC until use. In this study, the sensitivity and specificity of P29 antigen were evaluated (15).

Results

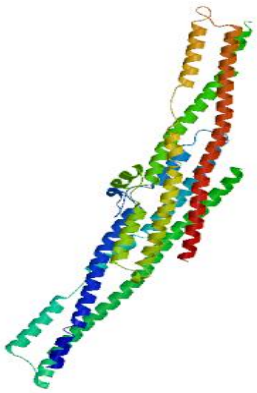
The recombinant DNA was first sequenced and aligned to other P29 sequences registered in GeneBank under accession numbers XP_024351425.1, AHA85396.1, CDS21096.1 and CAE1271104.1 using BioEdit ClustalW multiple Alignment program. The P29 sequence in this study showed 100% identity with P29 sequences derived from *E. granulosus* (XP_024351425.1), E. multilocularis (AHA85396.1), Endophilin B1 protein of *E. granulosus* (CDS21096.1) and 27% homology with peptide derived from Sepia pharaonis (CAE1271104.1) as an example of far phylogenetic tree species. The full Alignment result is not shown. Analysis the first (linear) structure of peptide using ProtParam online tool showed the total amino acid residues of 259 with the molecular weight of about 29 kD as expected. Lysine, Glutamine and Alanine are the most frequent residues of the peptide with the frequency percentage of 11.2%, 10% and 9.3% respectively (Table 1). Figure (1). shows the 3D prediction of P29 recombinant protein. Water molecular weight of recombinant P29 was calculated 18.015 according to the formula below; water Molecular weight = sum of individual residues weights - water molecular weight (number of residues - 1). Prediction of the P29 peptide motifs bound to MHC Class I/II binding sites were done using peptide analyzing tool to assist peptide design online program. P29 protein gel electrophoresis SDS-PAGE is shown in Figure (2). ELISA results of 25 human

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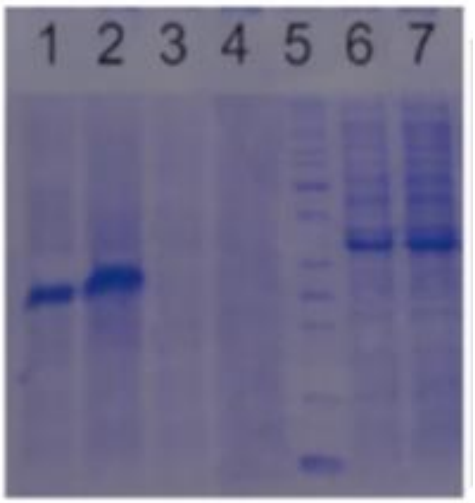
serum against recombinant P29 protein as Ag is shown in Figure (3).

Table 1. Frequency of amino acid residues in recombinant P29.

Amino acid		Number in recombinant P29	Frequency percentage
Ala	A	24	9.3%
Arg	R	10	3.9%
Asn	N	13	5.0%
Asp	D	17	6.6%
Cys	C	3	1.2%
Gln	Q	13	5.0%
Glu	E	26	10.0%
Gly	G	7	2.7%
His	H	9	3.5%
Ile	I	11	4.2%
Leu	L	20	7.7%
Lys	K	29	11.2%
Met	M	7	2.7%

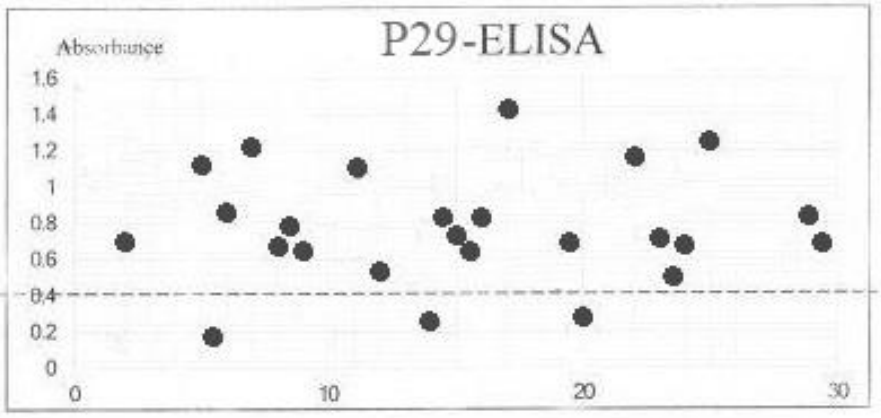


Figures 1. The third predicted structure of P29 recombinant protein with homology to SH3-containing GRB2-like protein 2 Crystal structure of the endophilin BAR domain mutant.



Figures 2. P29 protein gel electrophoresis SDS-PAGE. Well 1: *E. coli* Bl21-pET 28a-P29, well 2: *E. coli* Bl21-pET 28a- P2 ,10 µl, well 3: *E. coli* Bl21-pET 28, Well 4: 20 µl *E. coli* Bl21-pET 28a Well 5: Ladder, wells 6 and 7: Positive and negative control, respectively.

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Figures 3. The results of 25 human serums obtained from people harboring hydatid cysts evaluated by ELISA using P29 recombinant protein. Cut off = 0.4, Serum samples obtained from subjects.

Discussion

Hydatidosis is one of the most important parasitic diseases that arises highly emerging economic problems annually around the world (16).

Due to the use of different antigens in serological tests to detect hydatidosis (17) and the diagnosis of hydatid cyst using parasite-specific antigens as an immunogenic detective tool (15) but the usage of this method is reduced because of the cross reactions occured between antigens of hydatid cyst fluid and other parasitic diseases (18). Native antigens are extracted in from the corresponding source and show the native structure but recombinant antigens are instead manufactured artificially. A common method of making recombinant antigens is to transform the heterologous expression system with a vector expressing the protein of interest, following which the expressed protein can be purified from the culture broth. Regarding the recombinant antigens, although these may exhibit key structural differences to the native protein, with recent advances in protein engineering, they can now better mimic their native counterparts. Another aspect of using recombinant antigens in Immunogenic tests is that cross reactive-responses reduced by the recombinant antigens (19). Yuqing showed that Two different recombinant antigens derived from *E. granulosus*, *Echinococcus* protoscolex calcium binding protein 1 (rEg-EPC1) and thioredoxin peroxidase (rEg-TPx) were evaluated in this study to detect the specific immunoglobulin G (IgG) in sheep and goat with CE by the indirect enzyme-linked immunosorbent assays and the best diagnostic results were obtained in the anti-TPx IgG ELISA, with 92.6% sensitivity, 98.8% specificity, and no cross-reactivity with anti-Eg95 IgG (20).

The 12.8 KDa EPC1 antigen has a high reported sensitivity in detection the disease. (21). Recombinant Eg. P29 (rEg.P29) has a marvelous immunoprotection and can induce Th1 immune response (22).

In the present study, the recombinant DNA was first sequenced and comprised to other P29 sequences registered in GeneBank under accession numbers XP_024351425.1, AHA85396.1, CDS21096.1 and CAE1271104.1. The latest accession number was determined as a far species with low homology percentage rate. 25 human serum samples obtained from infected people who undergo to hydatid cyst surgery in Emam Khomeini Hospital, related to Tehran University of Medical Sciences were collected. Human serums with 5 different dilute of 1:10, 1:100, 1:200, 1:400, and 1:800 were analyzed based on indirect ELISA method

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using the prepared P29 recombinant antigen. Also, 20 negative control serums, from healthy individuals without Hydatidosis infection were utilized in the ELISA, mainly adolescents and children. For verification of tests, all subjects also were evaluated using an ELISA commercial kit (Pishtaz Medicine, Tehran, Iran). All 25 Hydatidosis serums reacted positively with P29 protein (Massih et al).In this study the P29 sequence comprised with somewhat similar sequences registered in Gene Bank and showed 100% identity with P29 sequences derived from *E. granulosus* (XP_024351425.1), *E. multilocularis* (AHA85396.1), Endophilin B1 protein of *E. granulosus* (CDS21096.1) and 27% homology with peptide derived from Sepia pharaonis (CAE1271104.1).This result confirms that P29 can be determined as a suitable candidate for designing serological detecting method of Hydatidosis with high sensitivity and specificity as expected that will decrease the cross reactivity with other parasites of the family Taenidae. Analysis of the second and third structure of the recombinant P29 showd homology to SH3-containing GRB2-like protein 2 BAR domain mutant. Analysis of peptide motifs which can be expressed in MHC-Class HLA of the immunologic system showed that P29 recombinant protein can be determined as a suitable Ag for vaccine development against Hydatidosis in future studies.

Conclusion

The recombinant P29 derived peptides can be produced and analyzed due to be determined as vaccine design candidate against Hydatidosis. The results may lead to be immune against other tape worm infections with Taenidae family.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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