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COMPARATIVE PROTEOMICS STUDY OF OUTER MEMBRANE PROTEINS FROM THREE PATHOGENIC LEPTOSPIRA SPECIES USED IN VACCINE

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ABSTRACT

Background and Objective: Outer Membrane Proteins (OMPs) have an important role in pathogenecity and immunogenecity of *Leptospira introgans*. The aim of this study was chemical and immunological analysis of OMP extracted from three vaccinal strains of pathogenic Leptospira (*L. canicola, L. grippotyphosa & L. sejroae hardjo*) by electrophoresis and western blotting.

Materials and Methods: Outer membrane enriched fractions that are insoluble in sodium N- lauryl sarcosinate isolated and studied by Sodium dodecyl sulfate – polyacrylamide gel electrophoresis. For identifying of antigenic properties of different proteins in three strains, antiserums developed in rabbit against the whole three valent vaccine and also specific OMPs rom each of three Leptospira strains. The antiserum was used for immunological studies with immuno double diffusion and western blotting.

Results: in SDS-PAGE five common protein bands with approximate molecular masses of 75,36,25,23 and 19 KDa were identified. After western blot studies identified four or five immunogenic band. In general, antiserum against L. grippotyphosa OMP has the highest ability in detecting common bands between three strains.

Conclusion: Chemical and immunological comparisons between OMPs of the three strains which are being used in the commercial vaccine, provided useful documents to assess and maybe improve vaccine quality. It indicated that OMPs are one of the major *Leptospira* component contributing in immunity and protectivity. Comparison between individual OMPs of each serotype revealed that *L. grippotyphosa* ones could contribute more in vaccine induced protectively in comparison with the other two serotypes.

Keywords: Leptospira; Outer Membrane Proteins (OMPs); Vaccinal Strains; Proteomics

INTRODUCTION

Leptospirosis is an important worldwide zoonotic disease of wild and domestic animals (1,2, 4,8,10). Clinical symptoms ranges from acute and severe (septicemia, hepatitis, nephritis, meningitis) to chronic (abortion, stillbirth, sometimes infertility, chronic nephritis, and recurrent uveitis), although sometimes infection is clinically unapparent .Genetic studies on *L. introgans*, have recognized that only some of leptospiral species cause pathogenecity of these bacteria. Isolation and identification species-specific antigenic protein of these bacteria could improve serological diagnostic, increase the vaccine quality and help to clarify the pathogenic involving mechanisms. It also could improve vaccination by providing cross- protective rather than serovar-specific, Lipopolysaccharide antigens. For this reasons, a number of other recent studies have also investigated the OMP of pathogenic Leptospires (1,2,3,5,6,7,8,9,10,11,12,13,14,15,16,17).

In this study we investigated antigenenicitically and structurally the OMPs of three pathogenic species used in a locally produced vaccine in order to evaluate the role of OMPs and also each species on immunological properties of this vaccine

2. MATERIALS AND METHODS

2.1. Leptospirals culture and preparation Of OMPs

The serovars used in this study acquired from microbiology department of Razi Vaccine and Serum Research Institute(RVSRI). The bacteria were initially isolated in EMJH (Ellinghausen & Mc cullogh) base medium with enrichment (rabbit serum) for 7 to 10 days.

After growth of bacteria in tube, strains were maintained by passage every two weeks in EMJH broth and incubated for four to seven days at 30˚c. Leptospires were then cultured at 30˚c in 750 ml EMJH broth to logarithmic phase of growth. Bacteria harvested by centrifugation. The pellet was washed twice in PBS(0.01 M phosphate – buffered saline (pH 7.2)), then was suspended in 10mM HEPES (N-2-Hydroxy-ethylpiperazine-N'-2-ethanesulfonic) buffer (pH 7.4).

The cells were disrupted by sonication. Cellular debris was removed by centrifugation at 1700 g for 10 minute .the supernatant fluid was centrifuged at 100000 g for 1 hour at 4˚c. The pellet was suspended in 3-5 ml of 1% sarcosyl in 10 mM HEPES buffer and incubated at room temperature overnight with gentle rotation. The insoluble fraction was

sedimented by centrifugation at 100000 g for 1 hour at 4˚c, suspended in 100 µl distilled water, and stored at -70˚c until doing SDS-PAGE.

SDS was also used as solublizing detergent beside sarcosyl,.

2.1. Development of antisera against OMPs

After OMPs extraction, protein mass was assayed with Bradford method. New Zealand white rabbits were injected subcutaneously with 1 to 1.2 ml of vaccine and OMPs which mixed 1:1 with Freund's complete adjutant on day 0 and with Freund's incomplete adjutant on day 21 and 42(booster doses were injected intramuscularly and subcutaneously). Serum was collected 7 days later and assayed with Immune double Diffusion (ID) and Microscopic Agglutination Test (MAT), to confirm antiserum against OMPs and Vaccine. The microscopic agglutination titers were 1:3200 using sejroae hardjo and 1:1600 for canicola & grippotyphosa strains. Serums were stored at -70˚c.

2.2. SDS-PAGE

OMPs enriched fractions were suspended 3:1 (V/V) in 4X sample buffer and boiled for 3-5 minute. undissolved material was removed by centrifugation.

Samples were separated in an vertical electrophoresis system (Paya Pajuhesh, Mashhad, IRAN) using 3% stacking and 10% resolving SDS-PAGE gels. After preparation of electrophoresis system and gels, samples were loaded in wells and run in 30 to 35 volt overnight.

Proteins were stained with coomassie blue and silver stain. The approximate molecular weight of each bands was then determined with plotting –logMW against Rf.

2.3. Gradient Gel method

In this method, two 4% and 15% resolving gels were used to prepare 4-15% gradient gels. After 1 hour, 4% gel or Stacking gel was added. This gradient gel improved the isolation of OMPs on SDS-PAGE in comparison with constant gels

2.4. Immunoblotting

Proteins were also electro blotted onto nitrocellulose and PVDF (Poly Vinylidene Di Fluoride) using a wet electro transfer system(Paya Pajuhesh, Mashhad, IRAN).

The transfer buffer contained: 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% Methanol. After preparation of system, protein transferred to it. The system was incubated with 63 volt for overnight in 4˚c chamber.

After finishing this process, polyacrylamide gel was removed from nitrocellulose or PVDF and stained with coomassie blue and silver stain to confirm protein transfer from gel to nitrocellulose or PVDF.

The membranes were blocked with 3% Bovine Serum Albumin (BSA) solution in 2% Sodium azide and incubated 2 hours at room temperature with gentle agitation. After two times washing in PBS, primary antibody (prepared antiserums) witch was diluted 1:200 with 3% BSA in PBS, was added and incubated 1 hour at room temperature with gentle agitation. Then after 4 times washing, secondary antibody (goat anti rabbit antiboddy conjugated with peroxidase) at 1:500 dilution was added and incubated 1 hour at room temperature with gentle agitation. Now blot is ready for staining after 4 times washing. For membrane staining or detection, 3-3' Di Amino Benzidine substrate was used. After detection of bands, reaction was stopped with PBS.

2.5. Cross Removal of vaccine antiserum

Purified OMPs for each strain was mixed with antiserum against vaccine and incubated for 2 hours in 37˚c. The Mixture was centrifuged and supernatants were used for western blotting.

3. RESULTS

3.1. OMP Extraction

For OMP extraction several methods were used. For example at first, low volume of bacteria (20-50 ml) was sonicated directly without any centrifugation and then it was electrophoresed, but any band was not seen. After increasing volume and application some procedure, the exact method was acceded.

Suitable time for sonication was 8-12 min after evaluation with dark field microscope.

Also SDS was used beside sarcosyl.in this procedure after SDS-PAGE few band was seen.

3.2. *SDS-PAGE*

The molecular weights of bands for each of the three strains were calculated by comparison with molecular weight standard. After SDS-PAGE, for Sejroe hardjo 11 bands with approximate molecular masses of 15.8, 19, 19.5, 23.4, 24, 24.6, 25.7, 35.1, 56.2, 70.7 and 74.1 KDa ,for Canicola 13 bands with approximate molecular masses of 14.1,18.6,19.9,21.8,22.3,23.4,24.5,35.7,38.9,63.1,67.6,75.8 and 79.4 KDa and finally for Grippotyphosa also 13 bands with approximate molecular masses of 19.9,23.4,24.5,25.1,28.1,36.1,47.8,56.2,63.1,75.8,79.4,89.1 and 100 KDa was observed.

5-6 common bands with approximate molecular masses of 19.7, 23.4, 24.5, 36.1 and 75.8 KDa were seen between three strains.

2-3 common bands with approximate molecular masses of 63.1 and 79.4 KDa were seen in Canicola and Grippotyphosa but not in Sejroe hardjo. 2-3 common bands with approximate molecular masses of 15 and 70.7 KDa were seen in Canicola and Sejroe hardjo but not in Grippotyphosa. 2-3 common bands with approximate molecular masses of 25.1 and 56.2 KDa are seen in Grippotyphosa and Sejroe hardjo but not in Canicola.

Several bands with approximate molecular masses of 28.1, 47.8,89 and 100 KDa were seen only in Grippotyphosa but not in Canicola and Sejroe hardjo. Several bands with approximate molecular masses of 18.6, 21.8, 22.3 and 38.9 KDa were seen only in Canicola but not in Grippotyphosa and Sejroe hardjo and two bands with approximate molecular masses of 19 and 24 KDa were seen only in Sejroe hardjo but not in Grippotyphosa and Canicola.

More than 90 KDa band was not seen, only in Grippotyphosa was seen one band.

Analysis by SDS-PAGE of outer membrane protein-enriched sarcosyl–insoluble material for three strains has been shown in figure 1.

3.3. Antiserum

After OMPs extraction, protein mass was assayed with Bradford method. The concentration of OMPs after comparison with standard (BSA) was: 203 µg/ml for Canicola, 221 µg/ml for Sejroe hardjo and 198 µg/ml in Grippotyphosa.Two New Zealand white rabbits were injected for each strain(4 times in 21 days interval).several day's after first injection, hardness and inflation were seen in the back. Three days after third injection, serum was assayed with Immune Diffusion (ID) and Microscopic Agglutination Test (MAT), to confirm anti OMP. Three days after last injection, sampling was done and serum again was assayed with ID and MAT tests. Results for MAT test, after mixing prepared serum at 1:100 dilutions, were: 1:3200 for Sejroe hardjo and 1:1600 for Grippotyphosa and Canicola. Result for ID test in the case of Grippotyphosa is shown in figure 2.

3.4. Immunoblotting

Proteins at first were electrophoresed, and then transferred to nitrocellulose or PVDF.

For obtaining amount of antiserum, one strain (Grippotiphosa) was tested at different dilutions (1:25; 1:50; 1:100 and 1:200) and conjugated antibody also was evaluated at several dilutions (1:200; 1:300; 1:400 and 1:500). For antiserum or primary antibody, 1:100 dilution and for conjugated antibody, 1:400, gave best answers.

Immunoblot was done with antiserum against OMP for each strain and vaccine. In immunoblot of antiserum against Canicola's OMP with Canicola's OMP, 5 bands, with Grippotiphosa's OMP, 4 bands and with Sejroe hardjo's OMP, 4 bands and with vaccine, 3 bands were detected. In immunoblot of antiserum against Sejroe hardjo's OMP with Sejroe hardjo's OMP, 4 bands, with Grippotiphosa's OMP, 5 bands and with Canicola's OMP, 4-5 bands were detected. In immunoblot of antiserum against Grippotiphosa's OMP with Grippotiphosa's OMP, 4 bands, with Sejroe hardjo's OMP, 4 - 5 bands and with Canicola's OMP, 4-5 bands were detected.

In immunoblot of antiserum against Vaccine with Grippotiphosa's OMP, 3-4 bands, with Sejroe hardjo's OMP, 3 bands and with Canicola's OMP, 3 bands were detected. Result for immunoblot of antiserum against Canicola's OMP with OMP of three strains and with vaccine, is shown in figure 3.

3.5. Cross Removal Method

This method was used for OMPs with Vaccine. In immunoblot of Canicola and Sejroe hardjo's OMPs after removal with antiserum against vaccine, with OMPs (three strains), no band detected. In immunoblot of Grippotiphosa's OMP after removal with antiserum against vaccine, with Grippotiphosa and Sejroe hardjo's OMPs, no detected any band, but with Canicola's OMP: 1 band was detected. (Figure: 4)

4. DISCUSSION

The technique of sonication, different centrifugation, and selection of sarcosyl-insoluble OMP enriched fractions from pathogenic leptospiral species been applied to leptospires previously (11,) and also has been successfully used to identify and characterize OMPs of other Gram-negative organisms. (12,14,16,) and of spirochetes (13).

Sarcosyl is an anionic detergent witch selectively solubilizes cytoplasmic membranes of Gram negative organisms (17). After accessing to optimum conditions for SDS-PAGE, for each strain several bands was identified (as was mentioned in results). 5-6 common bands with approximate molecular masses of 19.7, 23.4, 24.5, 36.1 and 75.8 KDa were identified between three strains. Several differences were seen in electrophoresis pattern for three strains. For example in

Grippotyphosa 4 bands with molecular masses of 28.1, 47.8, 89 and 100 KDa was detected, witch these bands specially two last one(89 and 100)with high MW can sensitize immune system strongly and give better immunization or in the case of Canicola 4 bands with molecular masses of 18.6, 21.8, 22.3 and 38.9 KDa were identified and because they are not seen in others, it could be used for immunization in vaccine.Vivan et al (11) identified 7 common bands (18,24,35.5,42,59.5,66 and 77 KDa) witch 4 bands are similar with our research (19, 24, 35.5 and 77). And according to their approach, the band with molecular mess of 35.5 KDa witch was separated in our three strains could be axial filaments. The 62 KDa antigen witch we isolated in the case of Grippotyphosa and Canicola was reported by sakamoto et al (1985) as an immunoprotective protein similar to other bacteria (E. coli, pseudomonas aeroginosa).

With this research and structural comparisons between three strains, could improve vaccine quality.

For evaluation of immunization properties, isolated OMPs and one dose of produced vaccine in Razi Institute, were injected to rabbits.OMP concentrations for injection were set in 200-300 µg/ml witch sensitize immune system completely. To confirm anti OMP, serum was assayed with Immune Diffusion (ID) and Microscopic Agglutination Test (MAT). MAT titers were suitable, witch for Sejroe hardjo was 1:3200 and for Grippotyphosa and Canicola was 1:1600, that it means antiserum production in high titer.

Antibodies were used in western blot for evaluation of immunogenicity and immunogenic bands. After SDS-PAGE of OMPs and transferring to nitrocellulose or PVDF, immunoblat was done. Optimal conditions for transferring and its time was 6-8 hour at 63 volt in transfer tank and also optimal dilutions for produced antibody (antiserum) was 1:100-1:200 and conjugated antibody 1:400-1:500.

Vivan M et al (11) detected 6 common bands in immunoblot for three pathogenic leptospira.one bands with molecular mass of 60-64 KDa was identified in immunoblot for P_{64}.

In our research, in immunoblot Sejroe hardjo's OMP with antiserum against Sejroe hardjo's OMP, 4 principle immunogenic bands, with antiserum against Canicola's OMP, 3 principle immunogenic bands, with antiserum against Grippotyphosa's OMP, 4-5 principle immunogenic bands and with antiserum against vaccine, 3 immunogenic bands, were detected. Witch in this case immunogenic properties for antiserum against Grippotyphosa's OMP and Sejroe hardjo's OMP are high.

In immunoblot Canicola's OMP with antiserum against Canicola's OMP, 5 principle immunogenic bands, with antiserum against Sejroe hardjo's OMP, 4 principle immunogenic bands, with antiserum against Grippotyphosa's OMP, 4 principle immunogenic bands and with antiserum against vaccine, 3 immunogenic bands, were detected. Witch in this case immunogenic properties for antiserum against OMP of three strains are high.

In immunoblot Grippotyphosa's OMP with antiserum against Grippotyphosa's OMP, 4 principle immunogenic bands with antiserum against Canicola's OMP, 5 principle immunogenic bands, with antiserum against Sejroe hardjo's OMP, 5 principle immunogenic bands, and with antiserum against vaccine, 3-4 immunogenic bands, were detected. Witch in this case also immunogenic properties for antiserum against OMP of three strains are high.

For preparing specific antiserum and cross antibody removal, cross removal method was used. In immunoblot for this method, only one band was detected. Witch this band was related to Canicola's OMP. This band is very valuable. Because it could be used as an immunogenic and antigenic criteria in PCR or diagnostic kit designs.

After immunoblot and evaluation of immunogenicity and immunogenic bands, approximately was resulted that antiserum against Grippotyphosa's OMP with other strains and also by it self, has more immunogenic bands and in result has a more antigenicity and immunogenicity properties. Witch this principle should be considered in vaccine production.

In the case of other strains (Sejroe hardjo and Canicola) according to common bands between them and with Grippotyphosa, almost are produced well cross immunity for themselves and Grippotyphosa.

According to this research and access to data about outer membrane protein, it suggested that OMP only could be used in vaccine (especially human vaccines) production.

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Figure1. SDS-PAGE of OMP from each of Leptospiraserovar:. Lane1: Low molecular weight marker, lane2: Leptospiragripotyphosa. Grippotiphosa lane3: Leptospiracanicolaand lane4: Leptospirasejroehardjo sOMP plus lane5: Albumin.

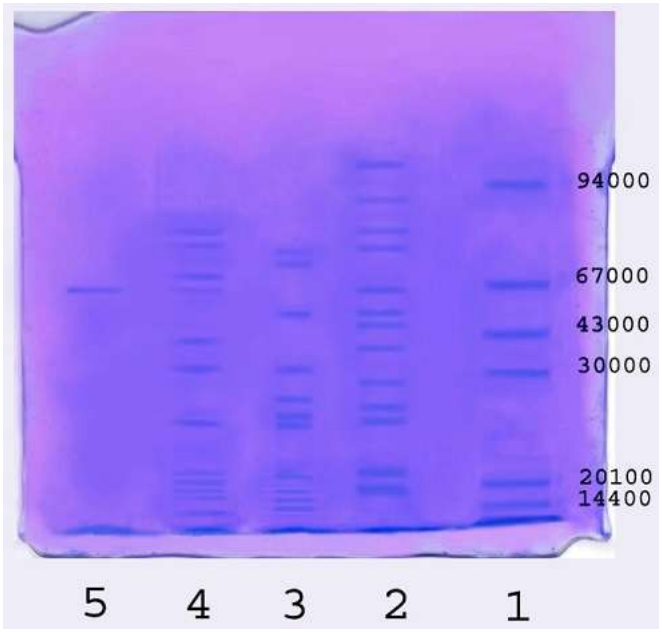


Figure2. Immunoblot of OMP extracted from laneV: Leptospira three valent vaccine preparation, lane C: Leptospiracanicola Lane G: Leptospira gripotyphosa Grippotiphosa and lane S:Leptospirasejroehardjo. The developed anti (three valent) vaccine was used as primary antibody and the rabbit conjugated to HRP as the secondary antibody.

Figure3. Immunoblot. Antibody cross removal. Lane1: sejroehardjo, lane2: canicola and lane3: gripotyphosa. Grippotiphosa's OMP, after removal with antiserum against vaccine. Only with Canicola's OMP 1 band was detected.

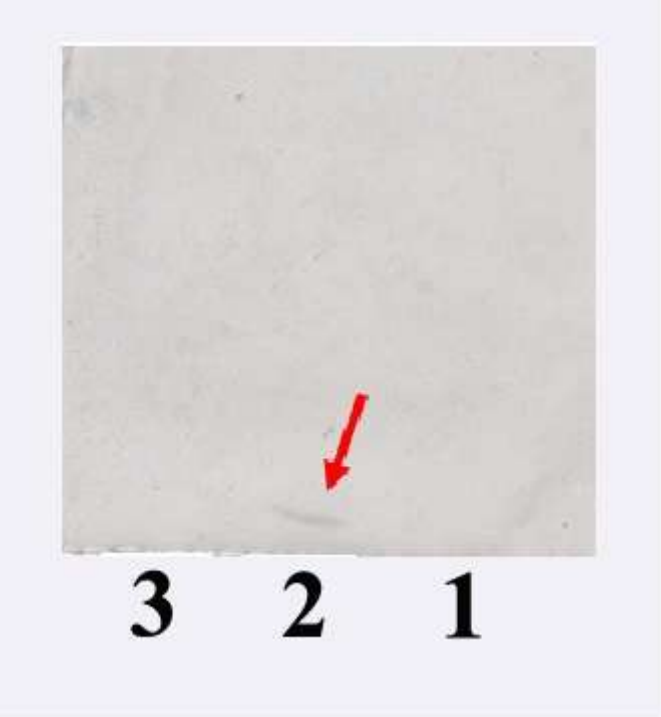
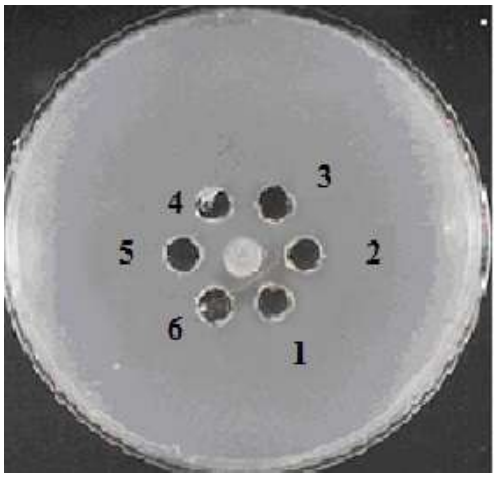


Figure4. Immune Diffusion midlle: OMP of Grippotyphosa, well 1: Anti OMP against Grippotyphosa with dilution of 1, well 2: Anti OMP against Grippotyphosa with dilution of 1:2,well 3: Anti OMP against Grippotyphosa with dilution of 1:4,well 4: Anti OMP against Grippotyphosa with dilution of 1:8,well 5: Anti OMP against Grippotyphosa with dilution of 1:16 and well 6: Anti OMP against Grippotyphosa with dilution of 1:32.



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3. Educations:

High school:

Navab Safavi 1984-1988.

University:

B.Sc. program: Faculty of science,School of chemistry, Tabriz university 1988-1992 (first class).

Project: A rapid spectrophotometric method for determination of adrenaline and nor-adrenaline in pharmaceuticals.

M.Sc. program: Department of biochemistry, Tehran medical sciences university 1993-1995 (first class).

Project: Production and purification of cholesterol oxidase and its application for determination of cholesterol in human serum.

Ph.D. program: Department of biochemistry, Tehran medical sciences university 1995-2000 (first class).

Project: Screening of asparaginase producing bacteria, expression and purification of asparaginase as an anti leukemia enzyme and study of its enzymatic and structural properties.

Post Doc Fellowship: Macromolecular assembly and cell signaling section, Macromolecular crystallography laboratory, National cancer institute at Frederick, National institute of health (NIH), Frederick, Maryland, USA, 1999-2001.

Projects:

1. Crystallography studies on Erwinia chrysanthemi asparaginase in order to determine mechanism of activity and substrate specificity.
2. Cloning, expression, purification, and site directed mutagenesis of Chemokines (MCP3, MCP5, Shinkine and Lymphotactine) for crystallography.

4. Professional experiences:

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1. Behesht Aein Laboratory Complex, 175, Marzdaran Blvd, Tehran-Iran, , 2005- present
2. Genomics and Genetic Engineering Department, Razi vaccine and serum research institute, Karaj, Tehran-Iran. 1997- present
3. Dentistry School, Azad University, Tehran-Iran, 2007-2009
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Some of Researchs I have been involved :

1. Large scale production of HA03 in Pichia pastoris for evaluation of as a recombinant vaccine (2015-2017)
2. Cloning and Expression of HA03 as a candidate anti tick recombinant vaccine (2009-2015).
3. Isolation and characterization of Bm86/95 analogue gene from Iranian Hyalomma anatolicum anatolicum as a candidate subunit recombinant vaccine (2004-2007).
4. Application of molecular modeling for identification of structural functional properties of asparaginase gene in different bacteria (2008-present).
5. Detection and Quantitation of measles virus by Real time PCR of F, H andN genes (2010-2013)
6. Genetic characterization RS-12, an Iranian isolates of Mumps virus (2005-2007)
7. Cloning and expression of fusion gene of AIK-C measles virus (2004-2006).
8. Application of (Epstein Barr virus) EBV for transformation of human lymphocytes in order to switch on telomerase gene (2003-2006).
9. Evaluation of telomerase activity in different cells (diploid and line) used for production of measles virus vaccine by TRAP-PCR.
10. PCR based detection and differentiation of pathogenic Leptospira serovars (2002-2005).
11. Cloning, expression, purification and site directed mutagenesis of human chemokines (MCP-3, MCP-5, Shinkine and Lymphotactin) (2000)
12. Crystallography of L-asparaginase as an anti leukemia enzyme (2000)
13. Screening asparaginase producing bacteria and study of the chemical properties of asparaginase isolated from different bacteria (1997-2000)
14. Research on development and stabilization of enzymatic kit for determination of cholesterol, glucose, urea, triglycerides and uric acid in serum (1994-1999).
15. Purification of peroxidase from Horse raddish for diagnostic application(1995-1997)
16. Production and purification cholesterol oxidase and its application for determination of serum cholesterol (1994-1996)
17. Determination of adrenaline and nor adrenaline in pharmaceuticals (1991).