Immunity of Surface Layer Protein of *Aeromonas hydrophila* in Rabbits

Lobna Adil Al-Noori **Frial Gemeel Abd** **Wejdan R. Taj Aldeen**
Department of Biology, College of Science, University of Babylon
loubna_adil@yahoo.com.

Abstract

In this study the Surface layer (S-layer) protein was extracted from *Aeromonas hydrophila* bacteria ,the humoral immune response that induced by S-layer protein only or as adjuvant was investigated by using 16 males New Zealand rabbits and divided into four groups, each group contained four rabbits, the first group was immunized with S-layer protein only, the second group was immunized with heated killed antigen(HKA)of *Sallmonella typhi* only, the third group was immunized with mixed antigens (S-layer+ HKA),while the fourth group considered as control group and immunized with normal saline. The HKA of *S. typhi* was used to evaluate the efficiency of S-layer protein as adjuvant. After the immunization period, the humoral immune response was investigated by several tests include, tube agglutination test and passive agglutination test that used to detect the antibody titer. Biuret method was used to determine the total protein concentration in serum samples and total protein concentration of secretory immunoglobulin that extracted form appendix samples. In addition the Radical Immunodiffution (RID) method was used to detect the concentration level of the IgG in serum samples. Moreover the concentration level of the CD4 in the serum samples was determined by enzyme linked immunosorbent assay (ELISA) method .In all these tests the result revealed, both of S-layer protein only , HKA of only and mixed antigens(S-layer+ HKA) were given significantly increased in comparison with control group at $P<0.05$. The result showed that the concentration level of IgG with mean values (2365.5 , 3505 and 2916) mg/dl respectively while the control group with mean value (1662)mg/dl. In addition the concentration level of CD4 molecule with mean values (9.37, 11.77 and 17.36) ng/ml respectively while the control group with mean value (6.91) ng/ml .The results showed that these three types of antigens induced the humoral immune response .

Key words: humoral immune response, surface layer protein, immune adjuvant.

الخلاصة

تم في هذه الدراسة استخلاص الطبقة السطحية البروتنية من بكتريا *Aeromonas hydrophila* وقد تم التحري عن الاستجابة المناعية الخلوية المحفزة بواسطة بروتين الطبقة السطحية لوحده او كمساعد مناعي باستخدام 16 ارنب قسمت الى اربعة مجاميع كل مجموعه تضمت 4 ارانب ,حيث تم حقن المجموعة الاولى ببروتين الطبقة السطحية فقط, اما المجموعة الثانية فقد حقنت بالمستضد المقتول للبكتريا *S. typhi* فقط ,والمجموعة الثالثة فقد حقنت بالمستضد المدمج المكون من بروتين الطبقة السطحية والمستضد المقتول ,اما المجموعة الرابعة فقد حقنت بالمحلول الملحي الفسلجي واعتبرت كسيطرة ,و تم استخدام المستضد المقتول لتقيم كفاءة بروتين الطبقة السطحية كمساعد مناع., وبعد انتهاء مده التمنيع تم التحري عن الاستجابة المناعية الخلطية من خلال عدة فحوصات, التلازن المباشر بالأنابيب والتلازن الدموي المنفعل لتقدير عيارية الاضداد. وقدتم استخدام طريقة بايوريت لتقدير تركيز البروتين الكلي في عينات المصل وتركيز البروتين الكلي للكلوبيولين المناعي الافرازي المستخلص من عينات الزائدة. بالإضافة الى ذالك فقد تم استخدام طريقة الانتشار المناعي لتقدير تركيز الكلوبيولين المناعي IgG في المصل, وايضا تم استخدام تقنية الامتزاز المناعي المرتبط بالأنزيم لتقدير تركيز CD4 في المصل, وقد بينت النتائج لكل الفحوصات ان كل من بروتين الطبقة السطحية فقط , المستضد المقتول للبكتريا *S. typhi* فقط والمستضد المدمج قد اعطت زيادة معنوية بالمقارنة مع مجموعة السيطرة وعلى مستوى احتمالية$P<0.05$ فقد كان تركيز IgG بمعدل (2365.5 ,2916,3505) mg/dlعلى التوالي بينما مجموعة السيطرة بمعدل (1662)mg/dl ,وقد كان تركيز CD4بمعدل(9.37,11.77,17.36)ng/ml بينما مجموعة السيطرة بمعدلng/ml (6.91) وقد اظهرت النتائج ايضا ان هذه المستضدات الثلاثة قد حفزت الاستجابة المناعية الخلطية.

الكلمات المفتاحية: الاستجابة المناعية الخلطية ,بروتين الطبقة السطحية, مساعد مناعي.

Introduction

S-layer protein are monomolecular arrays made up of a single protein species and represent the simplest biology membrane developed during evaluation,S-layer composed the outer most layer of the cell envelope of prokaryotic (archea and bacteria) (Sleytr *et al*., 2001). S-layer protein are produced by a number of pathogenic bacteria like *A. hydrophila* , *A. veronii* biotype *sobria, A. salmonicida* and *Campylobacter fetus* (Thompson, 2002).S-layer are generally composed of subunits of a single protein species. The S-layer is clearly an important virulence factor. The surface location and high copy number of the S-layer protein subunits also mean that the S-layer subunit protein is a major surface antigen (Dubreuil *et al*.,1990). While little is known concerning the antigenic structure of S-layers, in the case of the facultative intracellular pathogen *A. salmonicida* have S-layer proteins which are antigenically conserved (Kay *et al*.,1984). In contrast, a single strain of the extracellular pathogen *C. fetus* has the capacity to produce S-layer proteins of different antigenicities (Dubreuil *et al*.,1990). These *C. fetus* proteins also differ in their subunit molecular weights. S-layer-producing strains of *A. hydrophila* and *A. veronii* biotype *sobria* have the tetragonally arranged S-layers produced by these motile aeromonads. The antigenicity of the S-layers produced by pathogenic strains of *A. hydrophila* and *A. veronii* biotype *sobria* and report a third antigenic strategy of S-layer producing bacteria, while these S-layer-producing motile aeromonads all belong to a single lipopolysaccharide serogroup, and their S-layer proteins have similar subunit molecular weights (52kDa), the S-layer proteins display both NH2- terminal amino acid sequence diversity and antigenic diversity(Kostrizynska *et al.*,1992). One of the most relevant areas of research in nanobiotechnology is the technological utilization of self-assembly systems the natural assembly of S-layers into large regular arrays endows them with immune-stimulating and intrinsic-adjuvant properties(Hollmann *et al*.,2010)

S-layer carrier conjugates are superior vaccine carriers because they elicit DTH and immune protective antibody responses without the use of extraneous adjuvants and they can be administered by several different immunization routes (intramuscular, subcutaneous, nasal/oral), and they are immunological unique, which means that antibody and delayed-type hypersensitivity responses to each S-layer are specific and not cross-reactive (Sleytr *et al.,* 2014).

Material and methods

Preparation of antigens

Heat killed whole cell bacterial antigens was prepared according to the method described by(Agren *et al*., 1998).

S-layer protein with concentration (1mg/ml) that was used in this study as antigen was prepared as the followed: it was extracted from *A.hydrophila* isolates according to the method of (Ray and Johnson, 1986) by using sodium dodecyl sulphate(SDS 0.05%) solution *A.hydrophila* isolate was reactivated, then 1ml of bacterial suspension was added to tryptose soy broth and incubated at 37ºC for 24hrs.Then the bacterial cells were collected by centrifugation at 6000 rpm for 10 min. The pellet of cells was suspended by phosphate buffer solution pH(7) and washed 3 times by centrifuge at 6000 rpm for 15 min. Then the pellet was suspended by 5 ml SDS 0.05% solution and incubated for 10 min at 37ºC .Then the bacterial suspension was centrifugation at 12000 rpm for 15 min at 4ºC ,the supernatant represent the crude extraction(CE) was used and kept in refrigerator at 4ºC .After that, it was precipitated from crude extraction (CE)

according to the method of (Harris, 1989) by using ammonium sulfate with saturation ratio 80%. The purified S-layer protein concentration was measured by Bradford method (Bradford, 1976).It is molecular weight was determined by SDS- Poly acrylamide gel electrophoresis (SDS-PAGE) according to the method of (Laemmli, 1970).

While mixed solution (S-layer protein +HKA) antigen was prepared by mixing equal amount of S-layer protein of *A.hydrophila* bacteria at concentration of 1mg/ml with Heated killed Antigen (HKA) of *S. typhi* bacteria at concentration of (9×10^8 CFU/ml).

Laboratory animals

In this study, *Oryctylagus conniculus* rabbits were used at age 3-5 mounths and at weight 1-1.5 kg .sixteen adult males rabbits (16)were used to detect immune response for antigens, they were divided into four groups, each group composed of four rabbits and it was kept in cages specialized for animals in laboratory animal house and left there for two weeks for adaptation with consideration of use clean food and water for animals during the period of experiment (Schneider *et al.*, 1990).

Immunization program

Table (1) The Immunization program

Type of antigen		**S-layer protein of *A. hydrphila* only**	**Heated killed antigen of *S. typhi* only**	**Mixed antigens (S-layer protein of *A. hydrphila* +HKA of *S. typhi*)**	**Normal saline**
No. of animal		4	4	4	4
Concentration of antigen		1 mg/ml	9×10^8 cell/ml	(S-layer protein Con.1mg/ml +HKA Con. (9×10^8 cell/ml)).	0.85%
No. of doses		One dose each week for 3 weeks.	One dose each week for 3 weeks.	One dose each week for 3 weeks.	One dose each week for 3 weeks.
Dose	**First week**	0.5ml Ag with 0.5 of Freund's complete adjuvant	0.5ml Ag with 0.5 of Freund's complete adjuvant	0.5ml Ag with 0.5 of Freund's complete adjuvant	1ml
	Second Week	1ml	1ml	1ml	1ml
	Third week	1ml	1ml	1ml	1ml
Administrations method		0.25 ml right intramuscular	0.25 ml right intramuscular	0.25 ml right intramuscular	0.25 ml right intramuscular
		0.25 ml left intramuscular	0.25 ml left intramuscular	0.25 ml left intramuscular	0.25 ml left intramuscular
		0.5 ml subcutaneous	0.5 ml subcutaneous	0.5 ml subcutaneous	0.5 ml subcutaneous

Tube agglutination and passive haemagglutination test

These tests were used to investigate the presence of antigenicity properties of immunoglobulin in serum and in secretory immunoglobulin of appendix against S-layer protein ,HKA only and mixed antigens (S-layer protein +HKA)(Garvey *et al*., 1977).

Measurement of total protein concentration

This test was done by Biuret method by using kite provided by manufactory company (Biolabo, France).

Determine the level of IgG by radical immunodiffusin (RID)

In this test the concentration of immunoglobulin (IgG) in the sera of immunized rabbits was determined by using radical immunodiffusion plate that was supplied with kit, according to the method of (Mancini *et al,*1965).

Determines the level of CD4 molecule test

In this study, the concentration of CD4 molecule in the serum of immunized animals was detecting by enzyme linked immunosorbent assay (ELISA) method. This test was done according to the methods recommended by Manufacture Company (Elabscience).

Statically analysis

Data were processed and analyzed by using Anova one way, and the Least Significant Difference (LSD) was used to determine the significant difference between the different factors in the tests by using the using statistical program SPSS 19 statical program and the results were expressed (Mean±S.D). P-Values below 0.05 were considered to be statistically significant (George *et al.,* 2011).

Results and discussion

Antibody titer

A- Systematic antibody titer

The results of these tests of the systematic antibody titer in serum samples were showed in the table(1) that the rabbit groups immunized with S-layer only, HKA only and mixed antigens had given the same systematic antibody titer with mean value (5120) and these results show that S-layer protein only was given high systematic antibody titer in compared with control and this indicated that the S-layer protein induced immune response and this is agreement with the result that obtained from other study (Kokka *et al.,*1992) that mention 1mg of purified S-layer protein of *A. hydrophila* induce immune response in the new Zeeland rabbits and produced polyclonal antibodies which were genospecific, reacting only against S layers produced by *A. hydrophila* strains and not those from *A. veronii* .The results also reveal that the rabbits group immunized with HKA only was given higher systematic antibody titer in compared with control and this agree with the finding that obtained by other study (Hosny *et al.,*2015) that mention the antibody response to heat killed whole cell antigen of *S. typhi* immunization in mice was significantly higher than control group. In additional the rabbit group immunized with mixed antigen was given higher systematic antibody titer in compared with control and this agree with the results that obtained by other study (Malcolm *et al*.,1993) when the oligosaccharide(one to eight repeat unit) derived from type 8 capsular poly saccharide of *Staphylococcus pneumonia* were coupled to non-cross linked to S-layer protein from *Bacillus alvei* CCM 2051 and injected into the mice, the conjugates comprising relatively small oligosaccharide haptens elicited good antibody responses as determined by enzyme

immunoassay. Serum from the mice thus immunized had immune protective properties as revealed in a serum of mice immunized with heated killed *Staphylococcus pneumonia* .

Table(1) The systematic antibody titer in serum samples of rabbit groups immunized with different type of antigens.

Type of antigen	No. of animals	systematic antibody titer Mean ± S.D
S-layer of *A. hydrophila* only	4	5120
HKA of *S. typhi* only	4	5120
Mixed antigens (S-layer+HKA)	4	5120
Control	4	20

B- Mucosal antibody titer

The results of these tests of the mucosal antibody titer in appendix samples show in the table(2) that the rabbit groups immunized with S-layer only, HKA only and mixed antigens had given the same mucosal antibody titer with mean value(512) and it was higher than the mucosal antibody titer of control group with mean value(2). This result agree with the result of other study (Beganovic´ *et al*.,2011) that reported the Swiss albino mice group immunized with purified S-layer protein of *Lactobacillus helveticus* M92 was given high level of S-IgA in compared with control, in addition the mice that immunized with mixed antigen composed of *L. helveticus* M92 and *Salmonella typhimurium* FP1 cells was given also high level of S-IgA in compared with control.

Table(2) The mucosal Antibody titer in appendix samples of rabbit groups immunized with different type of antigens.

Type of antigen	No. of animals	mucosal Antibody titer Mean ± S.D
S-layer of *A. hydrophila* only	4	512
HKA of *S. typhi* only	4	512
Mixed antigens (S-layer+HKA)	4	512
Control	4	2

Total protein concentration

A -Total protein concentration in serum samples

The results show in the table(3)that there was significant increase in the total protein concentration in serum samples of the rabbit groups immunized with S-layer only, HKA only and mixed antigens with mean value (69.50, 77.30 and 72.32)g/L respectively in compared with control group with mean value (42.25) g/L at $P<0.05$ this agree with the finding of other studies (O'Brien *et al*.,2005) that mention that the purified S-layer protein of the clinical isolates of *Clostridium difficile* induced the humeral immune response and there was increased in the antibody titer of the serum sample of the rabbit that immunized with it.(Konstatinov *et al*. 2008) found that 45 kDa S-layer protein A from the surface of *L. acidophilus* was involved in the regulation of immature dendritic cells (DC) as well as cytokine production. The cellular contacts of DCs and *L. acidophilus* involve interactions between dendritic cell-specific intercellular

adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), a DC specific receptor DC-SIGN, and S-layer protein A, the dominant protein expressed by *L. acidophilus*

Table (3) The systematic total protein concentration in serum samples of the rabbit groups immunized with different type of antigens.

Type of antigen	No. of Animals	Protein Con. in serum g/ml Mean ± S.D
S-layer of *A. hydrophila* only	4	69.50 ±5.43*
HKA of *S. typhi* only	4	77.30 ±7.36*
Mixed antigens (S-layer+HKA)	4	72.32 ±4.87*
Control	4	42.25 ±3.30
L.S.D(0.05) = 8.39		

*Significant difference with control at P<(0.05).

B- Total protein concentration in appendix samples

The results show in the table(4)that there were significant increase in the total protein concentration of secretory immunoglobulin in appendix samples of the rabbit group immunized with S-layer only, HKA only and mixed antigens with mean value (63.30, 78.22 and 69.20)g/L respectively in compared with control group with mean value (45.87) g/L. This is agree with the fining of other study (Grogono-Thomas *et al.*,2003) that mention the S-layer protein induce the mucosal antibody response and he had used pregnant ewes (sheep) as modal animal experimental and immunized with wild type *Campylobacter fetus subsp. fetus* that expressed S-layer protein which caused abortion in the ewes, then it was challenged sub subcutaneously with wild type *C . fetus* that led to increase the level of S-IgA in the bile ,urine and milk.

Table(4)The local total protein concentration in appendix sample of the rabbit groups immunized with different type of antigens

Type of antigen	No. of Animals	Protein Con.in appendix g/ml Mean ± S.D
S-layer of *A. hydrophila* only	4	63.30 ± 3.87*
HKA of *S. typhi* only	4	78.22 ± 3.43*θ
Mixed antigens (S-layer+HKA)	4	69.20 ± 2.11*θ^
Control	4	45.87 ± 3.22
L.S.D(0.05) =4.974		

*Significant difference with control at P<(0.05). θ Significant difference with S-layer only at P<(0.05).^ Significant difference with HKA only at P<(0.05).

The concentration level of IgG immunoglobulin

The results show in the figure (5) that there were significant increase in the concentration level of IgG in the rabbit immunized with S-layer only ,HKA only, mixed antigen which were given mean values (2365.5 , 3505, 2916) mg/dl in compared with control group with mean value (1662)mg/dl at P<0.05.

The result revealed that the rabbits immunized with S-layer only was given significant increase in the concentration level of the IgG in compared with control, this agree with the finding of other study (Beganovic´ *et al*.,2011) that mention when the

mice immunized with the purified S-layer protein that extracted from *L. helveticus* M92 cells induced the immune response and the level of IgG, IgM and IgA antibodies were significantly higher in comparison to the levels of these antibodies in the control group of mice and in the group of mice immunized with *L. helveticus* M92 cells either with or without S-layer protein.

On the other hand the rabbits immunized with HkA only were given also significant increase in the concentration level of the IgG in compared with control, this agree with the results of other study (Yousif and Abd-Alkareem,2012) that mice had immunized subcutaneously with o.5ml of the somatic antigen (prepared by heat inactivation of *Salmonella ohio*) containing 1×108CFU the level of the IgG was significantly increased in compared with control at 2, 4, and 6 weeks in the immunized group post booster dose, and the maximum increase of antibody titers was determined at fourth weekIn addition the rabbits immunized with mixed antigen were given also significant increase in the concentration level of the IgG in compared with control, this agree with the results of other study (Malcolm *et al*.,1993) that the rabbits had immunized with conjugate vaccine composed of oligosaccharide of *Streptococcus pneumonia* that coupled with S-layer of *Paenibacillus alvei* was given high level of IgG in compared with control, and the oligosaccharide alone elicited the level of IgG response somewhat below the conjugate vaccine, but in the present study there was no significant difference between the mixed antigen in compared with HKA and S-Layer .

Table(5) The mean of the concentration level of IgG in serum samples in rabbit groups immunized with different type of antigens.

Type of antigen	No. of Animals	IgG con.mg/dl Mean ± S.D
S-layer of *A. hydrophila* only	4	2365.5 ±360.22*
HKA of *S. typhi* only	4	3505 ±341.56*$^{\theta}$
Mixed antigens (S-layer+ HKA)	4	2916 ±642.51*
control	4	1662±159.83
L.S.D(0.05) = 637.5		

*Significant difference with control at P<(0.05). $^{\theta}$ Significant difference with S-layer only at P<(0.05).

The concentration level of CD4 molecule

The results revealed as shown in the table (6)that there were significant increase in the concentration level of CD4 molecule in the rabbit groups immunized with S-layer only, HKA only and mixed antigen with mean values (9.37, 11.77 and 17.36) ng/ml respectively, in compared with control group with mean value (6.91) ng/ml at P<0.05 this is may indicate that the S-layer and mixed antigen take by phagocyte cell (antigen presenting cell) and presented by MHC II to CD4 T Cell (Th). This result agree with the finding of other study (Sleytr *et al*.,2014) that mention in cultures of peripheral blood mononuclear cells, both S-layer protein and (S-layer/Bet v 1) conjugate (but not rBet v 1) stimulated the production of high levels of IL-12, a pivotal mediator of Th1 responses. In addition, rabbits immunized with HKA only had significant increase the concentration level of CD4in compared with control, this agree with the finding of other study (Bergman *et al*.,2005)that reported CD4T-cell responses are an essential component of

immunity to Salmonellae. While the rabbits immunized with mixed antigen had significant increase in the concentration level of CD4 molecule in compared with rabbits immunized with HkA only this may be that the mixed antigen had more antigenicity than the HKA only.

Table (6)The CD4molecule in rabbit groups immunized with different type of antigens.

Type of antigen	No. of animals	CD4(ng/ml) Mean ± S.D
S-layer of *A. hydrophila* only	4	9.37 ± 0.65*
HKA of *S. typhi* only	4	11.77 ± 2.06*θ
Mixed antigens (S-layer+HKA)	4	17.36 ± 0.85*θ ^
Control	4	6.91 ±0.31
L.S.D(0.05) =1.794		

*Significant difference with control at P<(0.05). θ Significant difference with S-layer only at P<(0.05).^ Significant difference with HKA only at P<(0.05).

Conclusions

Immunization by S-layer protein only or as adjuvant induce humoral immune response

References

Agren, K.; Brauner, A. and Anderson, J. (1998). *Haemophilus influenza* and *Streptococcus pyogenes* group a challenge induce the Thl type of cytokine response in cells obtained from tonsillar hypertrophy and recurrent tonsillitis. OLR. 60: 35-41.

Beganovic´, J.; Frece, J.; Kos,B.; Lebos Pavunc, A.; Habjanic,k. andˇS usˇkovic, J. (2011). Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. Antonie van Leeuwenhoek Journal .100:43–53

Bergman, M.A.; Cummings, L.A.; Barrett, S.L.R.;Smith, K.D.; Lara, J.C. Aderem, A.; Cookson, B.T.(2005). CD4 T cells and toll-like receptors recognize *Salmonella* antigens expressed in bacterial surface organelles. Infection and immunity Journal. 73(3): 1350–1356.

Bradford, M.M. (1976).A rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing The Principle of Dye Binding. Analytical Biochemistry.72 (1-2):248-254.

Dubreuil, J.D.; Kostrzynska, M.; Austin, J.W. and Trust T.J. (1990). Antigenic differences among S-layer proteins of *Campylobacter fetus*. Journal of Bacteriology. 172:5035-5043.

Garvey, J.S.; Cremev, N.E. and Sussdrof, D.H. (1977). Methods In Immunology. 3th. ed. pp53-267. Addison-Wesley Publishing Company.

George, A.; Nancy, L.; Gene,W.; and Karen ,C.(2011).SPSS For windows Step by Step. Boston, Pearson Education. IMB. International Standard Book Number: 978-0-415-88229-3.

Grogono-Thomas, R.; Blaser, M.J.; Ahmadi, M. and Newell, D.J.(2003). Role of S-Layer protein antigenic diversity in the immune responses of Sheep Experimentally

Challenged with *Campylobacter fetus subsp. fetus*. Infection and Immunity Journal. 71(1):147-154

Harris, E. (1989). Concentration of Extract in Protein Purification Methods E.L.V. Harris and S. Angal .Oxford Uni.Press:154.

Hollmann, A.; Delfederico, L.; Miyoshi, A.; Disalvo, A.; De Antoni,G. Semorile, L. and Azevedo, V. (2010). S-layer proteins from *Lactobacilli* as vaccine delivery systems. International Journal of Microbiology Research.2:30-43.

Hosny, A. E. Diab; M.R.; Khattab, R.A. and Awad, H.O. (2015).Immune response to Vi polysaccharide, heat-killed whole cells, and outer membrane protein of *Salmonella typhi*. Infect Dev Ctries . 9(6):642-649

Kay, W.W.; Phipps, B. M.; Ishiguro, E.E.; Olafson, R.W. and Trust T. J.(1984). Surface layer virulence A-proteins from *Aeromonas salmonicida* strains. Can. J. Biochem. Cell Biol. 62:1064-1071.

Kokka, P.M.;Vedros, N.A. and Janda, J.M.(1992).Immunochemical analysis and possible biology role of an *Aeromonas hydrophila* surface array protein in septicaemia. Journal of General Microbiology.138:1229-1236.

Konstantinov, S.R.; Smidt, H.; De Voc, W.M.; Bruijns, S.C. M.; Singh ,S.K.; Valence, F.; Molle, D. ;Lortal, S.; Altermann, E. ;Klaenhammer, T.R. and van Kooyk, Y.(2008). S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci U S A. 105(49): 19474–19479.

Kostrzynska, M.;Dooly, S.G.; Shimojo,T.; Sakata, T. and Trust,T.J.(1992). Antigenic diversity of the S-Layer proteins from pathogenic strains of *Aeromonas hydrophila* and *Aeromonas veronii* Biotype *Sobria*. Journal of Bacteriology 174(1) 40-47.

Laemmli, U .k. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature .227: 680 - 690.

Malcolm ,A.J.; Best, M.W. and Szarka ,R.J .(1993). Surface layers from *Bacillus alvei* as a carrier for a *Streptococcus pneumoniae* conjugate vaccine. In: Beveridge, T.J. and Koval, S.F. Advances in Bacterial Para crystalline surface Layers. New York: Plenum.219-333.

Mancini, G.; Carbonaro, A.O.; Heremans, J. F. (1965). Immunochemical quantitation of antigens by single radial immunodifution. Immunochemistry, 2:235-54

O'Brien ,J.B.; McCabe ,M.S. Athie-Morales, V.McDonald ,G.S.;Ni Eidhin ,D.B.and Kelleher, D.P. (2005) .Passive immunisation of hamsters against *Clostridium difficile* infection using antibodies to surface layer proteins. FEMS Microbiol Lett .246: 199–205.

Ray, B., and Johnson, M.C. (1986). Freeze-drying injury of surface layer protein and protection in *Lactobacillus acidophilus*. Cry letters. 7: pp.210-217

Schneider, E.; Volcker, G.; and Hsude, W. (1990).Age and set dependent on phospholipid concentration in human erythrocyte. L.Z. Med .lab .Diag. 31:86-89

Sleytr, U.B.; Sara, M.; Pum, D.and Schuster,B. (2001).Characterization and use of crystalline bacterial cell surface layers .Progress in Surface Science. 68: 231-278.

Sleytr, U.M.; Schuster ,B. ;Egelseer, E. and Pum,D.(2014). S-layers: principles and applications. FEMS Microbiol Rev 38 :823–864.

Thomas, S. R. and Trust, T. J. (1995). Tyrosine phosphorylation of the tetragonal paracrystalline array of *Aeromonas hydrophila:* molecular cloning and high-level expression of the S-layer protein gene. J. Mol. Biol. 245:568–581.

Thompson, S.A.(2002). *Campylobacter* Surface-Layers (S-Layers) and Immune Evasion. NIH Public Access. 7(1): 43–53.

Yousif ,A.A. and Abd –Alkareem, H.A.(2012). Immune efficacy of *Salmonella ohio* somatic antigen in mice. MRVS 1(1):17-24.