Post-vaccination studies on mice vaccinated against uropathogenic *Escherichia coli*

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ABSTRACT

Vaccination is one of the most important strategies for fighting infection. The efficiency of vaccination is determined by many tests that evaluate the immune response vaccine elicit. Here Uropathogenic E. coli vaccines were evaluated by challenge test, indirect haemagglutination test, histopathology, haematology and biochemistry measurements. The results indicate to the benefit of inactivated polyvalent whole cell vaccine and adjuvant-use in immunopotentiation of vaccine. Many measures were used as a good indication and correlated to effectiveness of vaccine as post–vaccination antibody titer, total leukocytic count, neutrophil percent and total protein. Also the histopathology results for dead mice give clear clue to the interpretation of challenge test results.

Key words: albumin, antibodies, leukocytes, monovalent, polyvalent, protein, vaccine.

INTRODUCTION

Uropathogenic *Escherichia coli* is the single most common and important organism, accounting for approximately 85% of acute *cystitis* and *pyelonephritis*, as well as for more than 60% of recurrent *cystitis* and at least 35% of recurrent *pyelonephritis*. With the increasing problem of resistance in pathogenic microorganisms, the development of non-antimicrobial therapies is important. The use of vaccines against infectious diseases has been one of the true success stories of modern medicine. To date, over 25 vaccines have become available for human use. Adaptive immune response to uropathogenic *Escherichia coli* will protect the bladder from reinfection and whole urinary tract. Vaccine provides the most benefit to sexually active women who are in the 20 to 50 year- old age and have frequent UTIs. Intraperitoneal and intravesical immunization with killed bacteria stimulated local immune response in the urinary tract and protected against ascending UTI. After vaccine application many responses and variants can be measured, most common, challenge test and antibody titer measurement. Less used other tests, so, in this study we prepare many inactivated *E. coli* vaccine types prepared from UPEC and predict the elicited immune response in groups of mice. Variants of immune response and challenge, haematological, histopathological, and biochemistry analysis were examined post-vaccination in serum of examined mice.

MATERIAL AND METHODS

Bacterial isolation and identification

Three bacterial samples were collected from mid-stream urine samples of patients infected with urinary tract infection (UTIs are defined when establish a population of $> 10^5$ bacteria/mL of urine). Complete identification including most important biochemical tests was done, and serotyping for O antigen determination was carried out at the central laboratories of Ministry of Public Health. According to instructions of manufacturer Denka Seiken Co. LTD., with panels of polyvalent and monovalent antisera.

Vaccine preparation

The strain of monovalent vaccine was isolate of antigenic formula O78. The strains of polyvalent vaccine were isolates of antigenic formulas (O78, O114, O164). Preparation method was as reported by Russo et al., Ficken et al., Kaijser et al. and Li et al. Alumhydroxygel adjuvant (Rehesis New Jersy, USA) was added to some vaccine as illustrated below (19, 8, 10, 13).

Immunization and challenge

After one week of adaptation with new housing, 70 mice were grouped according to vaccine type into seven groups, each group contain ten mice: group 1 was given monovalent formalin-killed cells, group 2 was given monovalent formalin-killed cells with adjuvant Alumhydroxy gel, group 3 was given polyvalent formalin-killed cells, group 4 was given polyvalent formalin-killed cells with adjuvant Alumhydroxy gel, group 5 was given monovalent cell-free culture filtrate, group 6 was given polyvalent cell-free culture filtrate, and group 7was kept as a control group inoculated with PBS. Each mouse was inoculated intraperitoneay with 0.2 ml of the vaccine according to the following schedules: the first dose was inoculated at 0 day as initial dose, the second dose was inoculated at 7 day, and third dose was inoculated on 14th days. Blood collection with heparin as anticoagulant for antibodies determination was done at 7, 14 and 21th day post vaccination via intracardial route for one randomly selected mice for each group and with heparin as anticoagulant, plasma separated and kept at $-80^{o}C$ till used. Challenge test was done at 21th day post vaccination. All mice either vaccinated or control group were challenged with 0.2 ml contain 1×10^8 bacterial cell per mouse. Challenge dose was contained a mixture of virulent live UPEC (3 homologous strains) from which vaccine was prepared. Survival is monitored within 2 days after challenge.

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Indirect haemagglutination test

Antigens were prepared according to Kwapinski with modifications. Briefly, thick cell suspension of *E. coli* in phosphate buffer saline pH 7.2, was subject to the oscillation at 100 kHz with an audio frequency current of about 1.2 A and 220 W, with Sonicator (Fisher sonic Dismembra model 300), for 20 seconds in 5 pulse. Sheep erythrocytes were washed and tanned with tanic acid, then sensitized with antigen to be used in indirect haemagglutination test.

Haemagglutination test according to Boyden's technique as follow, series of two fold dilution of inactivated, erythrocyte-absorbed antiserum have been made in microtiterplate. Red blood cells are added in equal volumes of diluted serum about 0.250 ml. Control contain equal volumes nonsensitized red cells and a buffered saline. The plate is shaken for 10 minutes, then left at room temperature for approximately two hours. The mixture is incubated at $37^{o}C$ for 2 hours and left either at room temperature for 2-4 hours or at $4^{o}C$ overnight.

Total and differential leukocytic count

Total and differential leukocytic count was performed according to Brown. From well mixed whole blood 10 μl was added to 190 μl of lysing reagent (20.75 g NH_4Cl, 2.5 g $NaHCO_3$, and 0.093 g Na_2EDTA dissolved in one liter of distilled water), mixed and incubated for 1 minute. About 10 μl of mixture was transferred to haemocytometer chambers. Number of WBCs was counted in 4 large squares. Then calculate the total count per μl of blood after get rid of dilution factor. Differential count was done from thin blood smear, that fixed with absolute methanol, and then stained with Giemsa stain (diluted 1:4 with water) for 50 minutes and examined under oil immersion lens. Different types of WBC can be represented in percent from the total count.

Total protein and Albumin

Total protein was measured according to the instruction of the manufacturer (Bio-diagnostic com. Egypt).

Histopathological investigation

After challenge, the group of mice that exhibit more than 50% mortality rate after 10 hours have been abdominally dissected to get both kidneys and spleen from one dead mice of each group, and processed according to, and stained according to with hematoxylin and eosin.

Statistical analysis

Statistical analyses were done with SPSS software. With this software also Friedman has been used for challenge results, and paired sample T test for antibody response. Correlation used test was Spearman test of software SPSS.

RESULTS

Challenge test

Protectivity of vaccine in challenge test was calculated from deaths after 48 hours, Table (1). The highest protective index (PI) was for group 4 (polyvalent whole cell vaccine with adjuvant). Groups 3 (polyvalent whole cell vaccine without adjuvant), 2 (monovalent whole cell vaccine with adjuvant) and 1 (monovalent whole cell vaccine without adjuvant) with a percentage 57%, 50% and 40% respectively. They are good in comparison with group 5 (monovalent cell-free culture filtrate) and group 6 (polyvalent cell-free culture filtrate) which give low PI of 20% and 25% respectively. Statistical analysis for protection index (PI) values with Friedman test were showed significant difference between all vaccinated and control groups at value of P< 0.009.

Table (1): Protective index of different UPEC vaccine applied on mice with live virulent one.

Groups***	Death/Total	death%	PI*
1	3/5	60	40
2	3/6	50	50
3	3/7	43	57
4	1/5	20	80
5	4/5	80	20
6	3/4	75	25
C	5/5	100	0

[*]PI = Protective index (PI=[(% d of control-%d of vaccinated) / %d of control] x 100, *** Gr. 1 is monovalent (O78:K80) without adjuvant, Gr. 2 is monovalent (O78:K80)with adjuvant, Gr. 3 is polyvalent (O78:K80, O114:K90, O164K-) without adjuvant, Gr. 4 is polyvalent (O78:K80, O114:K90, O164K-) with adjuvant, Gr. 5 is monovalent(O78:K80) cell-free culture filtrate, Gr. 6 is polyvalent (O78:K80, O114:K90, O164K-) cell-free culture filtrate, and Gr. C is control.

Histopathology

Histopathological examination of Control mouse kidney was seen with marked congestion of glomeruli and increase cellularity, and rare neutrophil as always seen in acute glomerulitis. There was moderate intertubular congestion and focal haemorrhage. Its spleen was seen with depletion of lymphoid follicles, some necrobiotec changes in cells with increase megakaryocytes and rare neutrophil. For group 5 mouse vaccinated with monovalent cell-free culture filtrate and challenged 21th day post vaccination, its kidneys show milder focal haemorrhage than control. Degenerative changes (nephrosis) in renal tubules, with renal cast also noted. It was shown congested glomeruli with mild increase in mesengial cells. Kidneys of mouse group 6 was shown as that of group 5, but less in severity, degenerative changes are mostly vacuolation. Spleen of both mice was congested, mild haemosidrosis, with rare neutrophils and mild depletion in lymphoid follicles.

Antibody response to vaccination

Generally, anti-*E. coli* antibodies level was increased with the increasing number of dose to reach its maximum measured level at the second day after challenge for all groups (Figures. 1-3) (Table 2). Some groups reach that level at the third dose of vaccination. While the control group keep the same level of antibodies along the time of the experiment. Specificity of the humoral immune response have been seen obviously with more increase in level of anti-monovalent antibodies values in monovalent-vaccinated groups(group 1, 2, and 5) than polyvalent- vaccinated groups(group 3, 4 and 6). The same thing was true for anti-polyvalent antibodies values with polyvalent-vaccinated groups. Groups 2 and 4 that received adjuvanted vaccine, showed great increase in specific antibodies level after first and second dose. This note was most prominent for polyvalent-vaccinted groups than monovalent one. In addition they exhibit strong response against polyvalent antigens early. Groups 5 and 6 were show the lowest humoral immune response in comparison with other vaccinated groups but show specific response according to the type of vaccine they receive. Paired sample T test one tail analysis of difference between all mice groups with each other show significant difference with value of $P<0.05$ between the following groups:

In monovalent antigen test: group1 and group5, group1 and group6, group1 and control, group2 and group3, group2 and 6 and between group 2 and control. In polyvalent antigen test: group2 and group3, group2 and group4, group 3 and 5, group3 and 6, group 3 and control, group 4 and 5, group 4 and 6 and between group 4 and control. Group 3 and 4 show highly significant difference with control group.
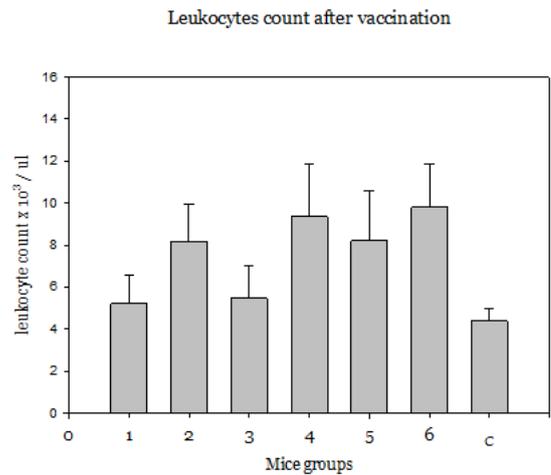

Figure (1): Mean Leukocytic count after vaccination.
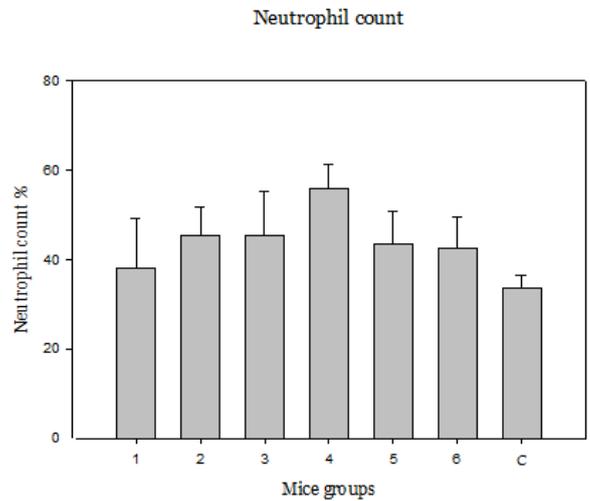
Figure (2): Mean leukocytic count after vaccination.
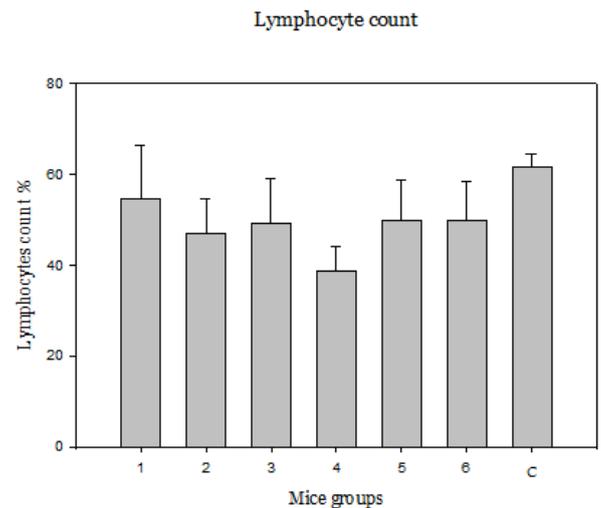
Figure (3): Mean leukocytic count after vaccination.

Table (2): Anti-*E. coli* antibodies titer measured with indirect haemagglutination test after each vaccine dose and post challenge.

Group of mice	Antigen used	Antibodies titer			
		7th day	14th Day	21th day	2nd day post-challenge
1	Monovalent	160	320	1280	1280
1	Polyvalent	80	320	1280	1280
2	Monovalent	80	640	640	1280
2	Polyvalent	40	160	320	1280
3	Monovalent	20	80	80	640
3	Polyvalent	640	640	1280	1280
4	Monovalent	5	80	320	320
4	Polyvalent	640	1280	1280	1280
5	Monovalent	20	80	320	640
5	Polyvalent	40	80	80	1280
6	Monovalent	20	40	80	160
6	Polyvalent	20	20	40	320
control	Monovalent	20	20	20	-
control	Polyvalent	20	20	20	-

Total and Differential leukocytic count

Figure (1) shows histogram representation of mean total leukocytic count for each group with standard error. From this figure, generally all groups show increase in total leucocytic count in comparison with control group with more elevation in group 4, group 6, than group 2, and group 5. The lowest elevation was seen with group1 and group 3, but still more than control group. Cell-free culture filtrate vaccinated groups (group 5 and group 6) show more increase in total leukocytic count than others, also adjuvant – received groups (group 2 and group 4) show more increase in total leukocytic count than others. In differential leukocytic count, mean neutrophil percent represented in figure (2) show elevation of neutrophil percent in all vaccinated groups than control one. There is less variation between groups in the degree of elevation, so the polyvalent adjuvant-received vaccine groups (group 2 and group 4) show the highest elevation with small standard error. In contrast with mean neutrophil percent, mean lymphocyte percent was shown for all groups below the level of control group as represented by histogram in figure (3). The lowest mean lymphocyte percentage was shown with group 4 (polyvalent adjuvant-received group). The highest mean lymphocytic percent within vaccinated groups have seen with group 1 with somewhat large standard error.

Total protein and albumin

Biochemistry investigation includes total protein and serum albumin with subsequently calculated globulin fraction and albumin/globulin ratio. Fibrinogen was neglected because of its very small quantity. From table (3) total protein show increase its amount with increasing the number of doses and reach its highest level in about all groups post challenge with an overall average of 5.4 g/dl. Albumin in contrast remains stable or around the overall average which was 3.8 g/dl. Globulin amount show increase levels in all groups with higher values after challenge in all groups than control. Albumin to globulin ratio show somewhat decrease or fluctuated results, but in comparison with a control group as a mean and standard deviation, as a general they was decreased and below that of control.

DISCUSION

Different types of vaccine that applied on mice yield different responses that reflect its efficacy. The protective index of the inactivated vaccine with adjuvant is better than vaccine without adjuvent. This enhances the rule of adjuvant in improvement of vaccine immune response especially if the vaccine itself is large-scale as the polyvalent vaccines used here. When adjuvant was added to this polyvalent one its effectiveness is stabilized. This illustrates the efficacy of adjuvant "Alum" in vaccine immunepotentiation. Totally whole bacterial-cell vaccine also good vaccine, it must be a target for development of new vaccine because the difference in effectiveness with cell-free culture filtrate was high. Whole bacteria possess natural adjuvant (LPS), and have the potential for development of bactericidal antibodies against conformational as well as linear epitopes. All mice of control group only showed severe urinary bleeding in the first hours of challenge while none of the others, including those of low PI this may related with effectiveness of vaccines in protection of UTIs, this also appeared in histopathological pictures. In my opinion the cause of death in vaccinated groups mainly due to the septic shock (with excess lipopolysaccharide), histopathological picture support this interpretation. Hisopathological examination of the dead mice from control group and cell-free culture filtrate group enhance this interpretation.

Table (3): Biochemistry investigations of mice groups vaccinated with monovalent and polyvalent UPEC vaccines and challenged with live virulent UPEC.

Time (day)	Group	Total protein	Albumin	Globulin	Albumin/globulin fraction
7th	1	4.5	3.4	1.1	2.9
7th	2	4.9	3.2	1.7	1.9
7th	3	6.0	4.4	1.6	2.7
7th	4	4.9	4.0	0.9	4.6
7th	5	4.8	3.9	0.9	4.1
7th	6	5.1	3.9	1.2	3.2
7th	c	4.9	3.9	0.9	4.3
14th	1	9.4	6.8	2.6	2.6
14th	2	4.2	3.4	0.8	4.3
14th	3	5.2	4.7	0.5	8.7
14th	4	4.3	3.9	0.5	8.2
14th	5	4.6	3.2	1.4	2.2
14th	6	5.3	4.4	0.9	4.8
14th	c	5.1	3.9	1.2	3.2
21th	1	5.5	4.2	1.3	3.2
21th	2	6.6	6.2	0.5	13.5
21th	3	5.2	3.8	1.4	2.7
21th	4	5.2	4.4	0.8	5.7
21th	5	6.0	4.3	1.7	2.5
21th	6	4.5	3.3	1.2	2.8
21th	c	4.6	3.9	0.6	6.2
Post challenge	1	5.1	4.0	1.1	4.0
Post challenge	2	7.5	2.6	4.8	0.5
Post challenge	3	6.0	3.3	2.7	1.2
Post challenge	4	4.9	2.3	2.6	0.9
Post challenge	5	6.1	2.7	3.3	0.8
Post challenge	6	5.3	2.9	2.5	1.2
Post challenge	c	5.3	2.7	2.6	1.1

The kidney of control mouse is of more severe picture than that of mouse in group 5 (monovalent cell-free culture filtrate vaccinated group) which in turn was severer than that of mouse group 6 (polyvalent cell-free culture filtrate vaccinated group). Severity of haemorrhage appears clinically as haematuria seen in non-vaccinated mouse while other didn't. This indicates acute nephritis and septic shock. Spleen changes during septic shock syndrome or after blood-born infection shown as acute congestion of the red pulp and efface the lymphoid follicles, neutrophils seen. Severe form appear with control mice which showed some necrobiotec change, this result demonstrate the benefit of vaccination. Humoral immune response measured by indirect haemagglutination test was gave a significant difference between different vaccines group as statistical analysis showing, all the mice produce protective antibodies after be evoked by vaccine. The highest elevation of antibodies titer showed with corresponding homologous vaccine group and less to heterologous one. This prove the specificity of the antibodies produced, but still good for other strains not included in the administered vaccine, this idea supported by high level of antibodies after challenge, so it can be regarded as protective vaccine. Immunopotentiation by adjuvant also improved antibodies response and enhanced with polyvalent nature of the vaccine used. This effect is clearly seen by comparing with cell-free culture filtrate vaccines. The effect of adjuvant was proved in many research, so many scientists use adjuvant with all vaccines type they used.

Haematological and biochemical tests that carried in this work reflect some of the immune responses. First of the all is total leukocytic count which represent important cellular immune response in inflammation after seven days of vaccination, in comparison with the normal range previously determined as published by Nemzek *et al.*, and Doeing *et al.*, our study revealed that the control group values were lie within normal value and all vaccinated groups show elevated values. More elevation caused by adjuvant which attribute to the slow release of vaccine by adjuvant and continuous elicitation of WBC. Highest elevation was noted with filtrate vaccine and mainly this may be due to the effect of high toxin content of the vaccine. All the vaccinated

mice in all groups have absolute neutrophilia (increased WBC and increased neutrophils), this happened as a result of inflammation elicited by vaccine. Another reason may relate to the origin of vaccine, i.e. it is bacterial vaccine. Lymphocytes mean value for the vaccinated groups was less than that of control. This strange result may attribute to the neutrophilia mentioned above. This make situation look like lymphocytopenia while this is not true, it may be normal because of normal response of antibody production.

The biochemistry analysis of vaccinated mice serum values reflects the immune response in the meaning of total antibodies which represent major globulin fraction of total protein. The gradual increase of antibodies production with increasing doses of vaccine to reach its maximum values after challenge reflected the values of globulin and consequently total protein. The most important value is the globulin value which comprises the antibodies produced in response to the vaccine. It showed increase level in all groups with highest values after challenge which showed by indirect haemagglutination test that gives compatible results. In contrast to that the values of globulin, the albumin globulin ratio were decreased with increasing doses of vaccination as a result of increased globulin and somewhat stable albumin. This also confirms the results of haemagglutination test.

Finally the correlations for all variants enhance the useful of its used collectively, so there was positive correlation between PI and antibody titer after vaccination, and neutrophil percent. As clearly seen the results of post-vaccination antibody titer and results of total protein and globulin calculation were parallel.

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