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IMMUNIZATION MICE WITH DNA FROM PROTOSCOLICE OF HUMAN HYDATID CYST: A IMMUNOLOGICAL STUDY

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ABSTRACT
The objective of this study was to investigate the ability of DNA antigen in induce an immune response in BALB/c mice against secondary hydatid disease and compared with native B antigen, using immunological approaches. Three antigens were extracted and giving in three doses and concentration (0.1,0.2,0.3 ml) with one month interval between each using two routes intramuscular and subcutaneous (I.M & S.C) to 54 mice (15 mice for each antigen) in the preliminary experimental work, these were chromosomal DNA, the yield of DNA purity was1.8µg/ml and the concentration was 223 µg/ml, recombinant plasmid DNA, the concentration and purity was 268,1.85 µg/ml respectively and the native B-antigen was partially purified from human liver hydatid cyst fluid, the protein concentration was 0.4 mg/ml. Humoral immune response was assessed by double diffusions test (DDT) and indirect haemagglutination test (IHA). Results were taken as a guideline in the challenge experiments. Immunization of 65 mice was conducted by IM&SC routes using these antigens at dose of 0.3 ml to be followed by injection mice intraperitoneally with a live protoscolicesa month later and to positive control. Humoral immune response was assessed by IHA test together with latex agglutination test conducted 120 days post-challenge. The recombinant plasmid DNA was highly efficient in resisting infection giving a 100% reduction rate of hydatid cyst followed by native B antigen 88.3%, 85.7% by I.M and S.C routes respectively. A significant differences ($p<0.01$) was found within and between the immunized groups and between the positive control. Antibody titers were high and increasing in the positive control and in the immunized infected groups reach 64 by IM and 32 by SC in DNA group and decline to 4 in recombinant plasmid DNA group while in the native B the titer reach 128 in both IM and SC group's. The results of latex test were variable. Cell-mediated immune response was detected after 3hours then decline and disappears after 24 and 48 hours in all groups.

KEYWORD: chromosomal, recombinant plasmid DNA, native B antigen secondary hydatid disease immunological study

INTRODUCTION

Radical change in vaccine methodology comes along-with advent of DNA-mediated immunization known as DNA vaccines, vaccine out of genetic material either DNA or RNA [1,2]. In 1992 studies revealed that DNA vaccines delivered into cells could stimulate the immune system of rodents and primates to generate B cells, cytotoxic T cells and T helper cells response against many different pathogens[3]. The research showed as well, that immune response and disease protection could be elicited when different routes of administration were used[4]. The responses, moreover, could be enhanced by a variety method for facilitating DNA uptake by cells [5].The earliest trial began in 1995, when plasmids containing human immunodeficiency virus (HIV) genes were delivered to patients already infected by that virus. Then physician put genes coding for HIV proteins into healthy people instead of in those affected by some disorder. So far human tests are examining vaccines designed to prevent herpes, influenza, hepatitis B virus and plasmodium, to bolster the impaired immunity of patients already infected[6]. Echinococcosis / Hydatidosis, is one of the world's major helminthes zoonosos caused by adult or larval stage of cestodes belonging to the genus *Echinococcus* and the family Taeniidae. The disease has a considerable economic and public health problem in many regions of the world and its global distribution is partly due to the ability of the parasite to adapt to a wide variety of domestic and wild intermediate and definitive hosts [7]. In Iraq, hydatidosis it is still a major economic and public health problem, as there is not yet an organized national control program. Disease is endemic and enzootic[8], many studies had been carried out on the disease from different aspects. On the molecular level, however, during (2002 - 2003) the time study performed only two studies had been conducted in Iraq, *viz*. [9] succeeded in constracted a gene bank of DNA from hydatid cyst of local isolates in the pBR328 plasmid, while[10] applied PCR technique in order to differentiate *Echinococcus* strains in north Iraq. A new approach in immunization have been applied for the first time, to investigate the effect of DNA as antigen induce an immune response against secondary hydatid disease in BALB/c mice depending on its effectiveness in preventing many diseases.

MATERIALS & METHODS

The solutions were used throughout this study according to[11]. The weight of each substance in the solution was measured according to the equation Weight = volume X molarity X molecular weight /1000. Samples collection : Hydatid cyst were obtained from lung and liver during operation from infected patients admitted to general

surgery wards of Baghdad teaching hospital. Intact cysts were transferred in cool boxes with sterile normal saline to laboratory of pathology and forensic medicine department medicine college of Baghdad. The cysts were immediately processed,aspiration of fluid collection,estimation viability and Counting of protoscolices (PSC) and the pellt stored in 70% ethanol[12].

Antigens preparation: Extraction and purification of the chromosomal DNA using enzymatic organic method of [12] from freshly collected psc and those stored in 70% ethanol. Recombinant plasmid DNA extraction and purification from recombinant colonies of *E. coli* k12-derived laboratory strain (MM294) stored in glycerol media at -20^{o} C under the name REC-296 represent a partial gene bank of DNA hydatid cyst were kindly supplied by[9]. Colonies of *E. coli* from the broth and those from control plate of *E. coli* strain obtained from department of microbiology of teaching laboratories in Baghdad Medical City, were subjected to alkaline lysis method[12]. Spectrophotometric measurement of UV absorbance of optical density (O.D) at wave length of 260 and 280 nm) as follow:

*DNA conc. (*µ*g/ml) = measured OD (260NM) x 50* µ*g/ml x dilution factor*. An OD 260 of (1) corresponds to 50 µg/ml of DNA. The ratio between the readings of (OD260/OD280nm) provides an estimate of the purity of DNA[12]. Gel electrophoresis was performed for DNA visulization [11]. Native B antigen and protein measurement according to [13,14].

Preliminary study: (Twenty five-30) day's old male BALB/C mice obtained from breeding colony of animals house of institute for research and infertility treatment. Three of mice were housed in separate cages under suitable environmental. The study conducted on 54 mice, divided into 3 principal groups according to antigen each included 18 mice each divided into 2 subgroups (IM) and (SC) each compose of 9 mice. In the first and second groups, DNA was given in repeated 3 doses of 1 and 2 weeks intervals .The first subgroups was immunized with 0.1 ml, the second was giving 0.2 ml, and the third 0.3ml in a concentration of 0.1, 0.2 and 0.3 mg/ml respectively .Native B antigen also giving in repeated dose of 0.1, 0.2 and 0.3 ml and in a concentration of 0.4 mg/ml for each sub groups, in the third dose the volume was reduce half the original one. A negative control group of 3 mice was giving normal saline. The animals were watched for any side effects. Serum was collected after one week of giving the 3rd dose.

Serological tests

1. Double diffusion Test (DDT) has been used to test the affinity of the antibodies to the prepared antigens [15].
2. *Anti-Echinococcus* antibodies were measured by indirect haemagglutination test (IHT)[16].

Design of the challenge experiments**.** Sixty five mice were used throughout the study. The mice were grouped into 3 categories each included[20] mice. These were subdivided into 2 subgroups (i.m and s.c) routs, each included 10 mice. The first group were immunized with chromosomal DNA at a concentration of 0.3 mg/ml in total volume of 300 µ l . After one week 150µ l (half the first dose) was mixed with the same volume of $CaCl_2$ solution and given as the second dose.

After 21 days half the original dose was given as a booster dose. The second groups were gave recombinant plasmid DNA in the same way. The third groups were gave B antigen in a concentration of 0.4 mg/ml and volume of 300µl in the same way. The forth group (5) mice represent positive control. After 8days from the final dose of immunization 0.5 ml of hydatid fluid containing 2000 psc were given intraperitoneally as challenge dose to all mice .Mice were dissected after 120 days post infection {dpi}. Serum collected divided into aliquot and kept at-20°C till use. The internal organs were examined looking for hydatid cysts to calculation the reduction percent (R.P) [12].

$$\text{Re}\,duction\,\% = \frac{Mean\ No.of\ \ cyst\ \text{in}\ \ positive\,control - Mean\ No.of\ \ cyst\,in\,immunized\ mice}{Mean\ No.of\ \ cyst\ in\ \ positive\,control} \times 100$$

Serological tests: Humoral immune response were assessed by IHA test [16] and Latex agglutination test (LAT)[17]. Cellular immune response was assessed by Delayed Type Hypersensitivity Test (DTH) [18].

Statistical analysis

Data estimated by T-test of two side differences at P<0.05, 0.01 were regarded significant [19].

RESULTS

Fresh intact lung cysts were obtained fertile, the packed volume of psc obtained ranged from 0.5-2 ml. The mean viability of the psc exceeded 90% when assessed by microscopic examination of flame cell activity and eosin exclusion. Native B antigen prepared from fluid of two liver cysts. The protein concentration was relatively high, 0.4 mg/ml, based on the linear relationship drown between (O.D) of different protein concentration of bovine serum albumin (Fig.1). DNA concentration and purity: A whitish pellet of chromosomal DNA appeared following centrifugation at 6000 rpm (Fig. 2). The purity of DNA was yield higher. Plasmid DNA extracted from both recombinant transformed and control colonies was in the form of whitish precipitate also. The concentration and purity for both were illustrated in, (Table 1).

Results of DDT : In the sera of mice immunized with B antigen the reaction area appeared as a whitish line of precipitate seen with the first and double dilution of antigen in the second well .Also between the antigen and of all sera obtained from immunized groups with different concentration and from both(IM) and (SC) routes (Fig. 3, 4).

Results of IHT: Its revealed a significant difference (P<0.05) between antigens .On increasing the dose to 0.3 ml, high levels of antibodies were detected in all sera obtained after one week from the 3rd dose with the all antigens using both routes (Table 2).

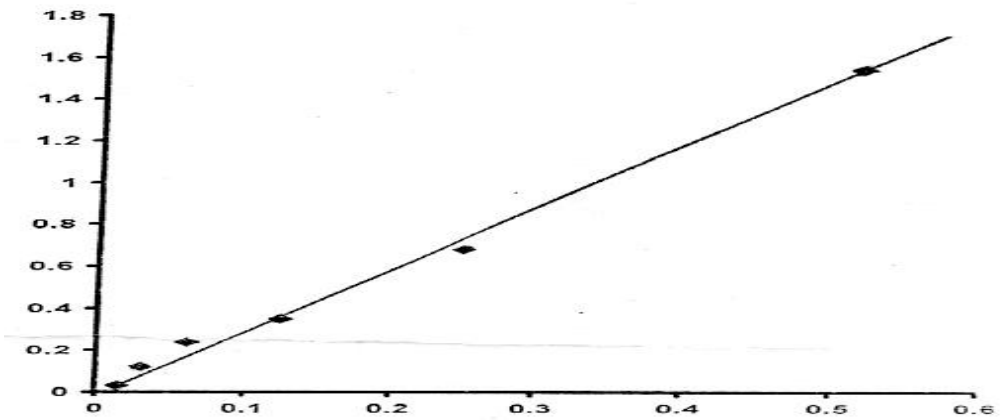

FIGURE 1: Optical density readings of different protein concentration of bovine albumin and B-antigen.

TABLE1: O.D ratio of concentration and purity of chromosomal and plasmid DNA.

Sample	O.D at 260 nm	O.D at 280 nm	Concentration µg /ml	Purity
Chromosomal DNA	0.223	0.121	223	1.84
Recombinant	0.268	0.145	206	1.85
Control	0.135	0.075	135	1.8

TABLE 2: Mean titer level of antibodies detected after one week of immunization.

Treated group	Intramuscular route						Subcutaneous route					
	0.1 ml		0.2 ml		0.3 ml		0.1 ml		0.2 ml		0.3 ml	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Chromosomal DNA	70.4	35.05	76.8*	28.6	102.4*	32.05	44.8	17.5	76.8	28.6	102.4*	32.05
Recombinant Plasmid DNA	152.8	55.4	179.2*	70.1	230.4*	166.8	115.2	28.6	140.8	70.1	204.8*	70.10
Native Antigen	51.2	17.52	89.6*	35.05	179.2*	70.08	73.6	52.5	69.8	35.2	115.2*	83.44

*Asignificant difference (P<0.05) between antigens

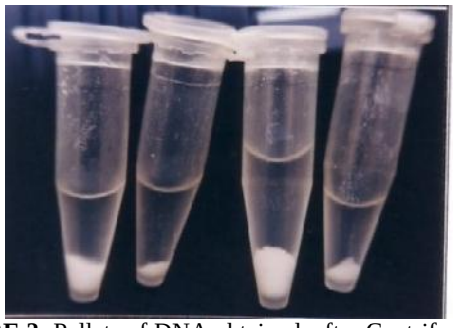

FIGURE 2: Pellets of DNA obtained a fter Centrifugation

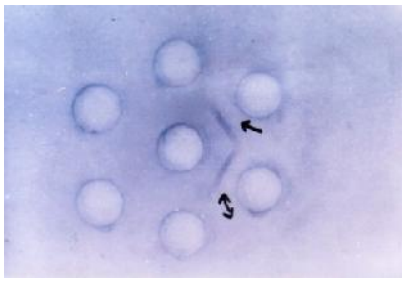

FIGURE 3: Double diffusion test a precipitateline between serum in the central well and first and econd dilutions of B-antigen (→) (↔).

Results of challenge: Inspection 5 mice, which represent positive control group, show that all were infected with secondary hydatid cysts (Table3). In mice immunized with chromosomal DNA the (R.P) was high 94.8% and 93.5% using im and sc routs respectively, using B-antigen the (R.P) was also high 88.3% and 85.7% in im and sc groups respectively. In case of animals immunized with recombinant plasmid DNA results revealed absolute resistant 100% to infection in both im and sc groups dissected 120dpi with significant differences (P<0.01) between positive control and immunized groups. (Table, 3)(Fig 5,6). No significant difference was detected between Im and sc routes.

Results of IHT: A significant difference (P<0.01) was found between antibody titers of positive control group and all immunized groups 120 dpi. A significant difference (P<0.01) was found also between native B antigen group on one side and low titers in chromosomal and plasmid DNA groups on the other side post 120day. On the other hand the difference in antibody titers between both routes was not significant (Table, 4).

Results of Latex test: Particle suspension (10%w/v) was used for preparing *Echinococcus* reagents. The size of the particles was 0.3µm. Tests were carried out after 120 dpi on 44 serum samples consist of (22 im, 22 sc) routes and 3 samples of negative control. The positive results were recorded in all seven samples (100%) of positive control. While the negative result was in all sera obtained from mice immunized with recombinant plasmid DNA by im and sc routs. In mice immunized with B antigen, test gave positive result only in 4 (50%) by im and 4 (57.5%) by sc (Table5). A positive serum shows clumps of latex particles

as complete aggregation, while a weakly positive reaction shows fine aggregation. Statistically, there is no significant differences between routes of injection, the significance (P<0.05) was between antigens notably the recombinant plasmid and others, also seen between immunized groups and positive control groups. Only 50% and around of the positive control and infected immunized mice revealed the presence of antibodies. The cysts in the positive control sacrificed 120 dpi were appeared either as a single large transparent having sterile fluid lying in the peritoneal cavity or as multiple cysts, in clusters adhered to the liver, spleen, the rest was free in the abdominal cavity (Fig. 3). In the immunized infected group, cysts were found few in the form of small nodules attached to internal organs in early stages of infection. They changed into opaque cysts in some instances on the surfaces unembedded in tissues (Fig, 3).

Results of DHT test conducted 120 dpi and before sacrificing the animal show a high increase in the footpad thickness in the positive control 3 hours post local injection of B-antigen then decreased after 24 and diminished after 48hours.Regarding the immunized mice; a high increase in footpad thickness was also noticed at the same periods (Table 6) with a significant difference (p<0.05) between the positive control and immunized mice.

TABLE 3: Number, diameter and (R.P) of cysts in mice dissected 120 dpi.

Treated Group	Intramuscular route						Subcutaneous route					
	No. of mice	No. of +ve	%	No. of cyst	Diameter of cyst (mm)	Reduction %	No. of mice	No. of +ve	%	No. of cyst	Diameter of cyst (mm)	Reduction %
Chromosomal DNA	10	2*	20	2 – 2 (4)	2 – 3	94.8	10	3*	30	1 – 3 (5)	2 – 4	93.5
Recombinant plasmid DNA	10	0*	0	-	-	100	10	0	0*	-	-	100
Native B Antigen	10	4*	40	2 – 3 (9)	2 – 4	88.3	10	4*	40	2 – 3 (11)	2 – 4	85.7
Control/ ip	5	10	100	77	2 – 8	0						

*Significant difference (P<0.05) between antigens and positive control

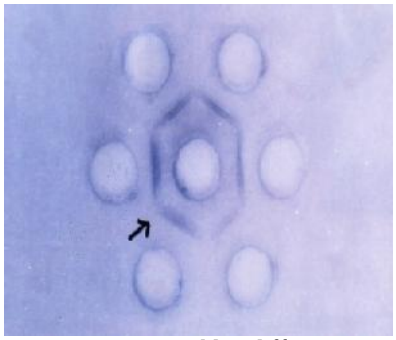

FIGURE 4: Double diffusion test between B-antigen in the central well and with sera from all immunized. A continuous line of precipitate is seen (→).

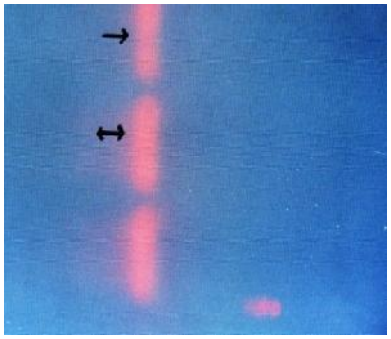

FIGURE 5: Bands of chromosomal DNA seen under uv trans-illumination. The gel rune from left to right. The voltage was 85 v.

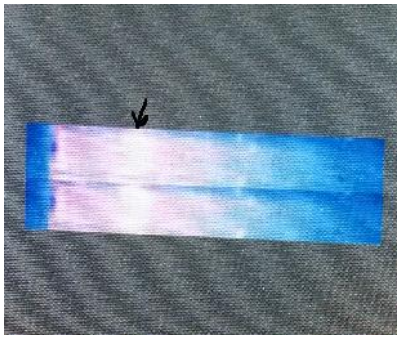

FIGURE6: Recombanant plasmid Seenunderuvtrans-illumination as single wide band

TABLE 4: The ranged and the mean of antibody titers and SD in all groups 120 dpi.

Treated group	Intramuscular route		Subcutaneous route	
	Mean	SD	Mean	SD
Chromosomal DNA	16 -64 (27.4)*	21.1	8 -32 (18.1)*	13.1
Recombinant plasmid DNA	2 – 4 (3.1)*	2.15	2 -4 (3.1)*	2.15
Native B antigen	16 – 128 (58)*	47.82	4- 128 (60.5)*	43.85
Positive control	128 – 256 (201.1)*	140.123	-	-
Negative control	-	-	-	-

*Significant difference (P<0.05) between antigens

TABLE 5: Results of latex agglutination test obtained 120 dpi .

Treated group	Intramuscular route			Subcutaneous route		
	No. of samples	Positive	%	No. of samples	Positive	%
Chromosomal DNA	7	3*	42.8*	8	3*	37.5*
Recombinant plasmid DNA	7	0	-	7	0	0
Native B Antigen	8	4	50*	7	4	57.1*
Positive control	7	7*	100*	-	-	0
Negative control	3	0	-	3	-	0

*Significant difference (P<0.05) between antigens

TABLE 6: The range, mean and SD differences in thickness of footpad (mm) 120 dpi

Treated groups	Intramuscular route after						Subcutaneous route after					
	3h		24h		48h		3h		24h		48h	
	Range (mean)	SD	Range (mean)	SD	Range (mean)	SD	Range (mean)	SD	Range (mean)	SD	Range (mean)	SD
Chromosomal DNA	2.7 – 3 (2.8)	0.317	2.2 – 2.9 (2)*	0.181	1.9 – 2.1 (1.9)*	0.073	2.3 – 2.9 (2.4)	0.196	2 – 2.5 (2.13)	0.163	1.9 – 2.1 (2)*	0.056
Recombinant plasmid DNA	2.5-3.2 (2.5)	0.207	1.9 – 2.5 (2.4)	0.218	1.9 – 2.1 (2)	0.105	2.3 – 2.9 (2.5)	0.216	2 – 2.5 (2.1)	0.193	2 – 2.1 (2)*	0.067
Native B antigen	2.5 – 3.1 (2.6)	0.399	2 – 2.5 (2.17)	0.231	1.8 – 2.5 (2)	0.188	2.3- 2.9 (2.6)	0.185	2 – 2.5 (2.2)	0.179	2 – 2.1 (2)*	0.067
Positive control\I.P	3.1 – 3.9 (3.4)	0.308	2.3 – 2.9 (2.5)*	0.217	1.9 – 2.5 (2.2)*	0.217	-	-	-	-	-	-

*Significant difference (P<0.05) between antigens

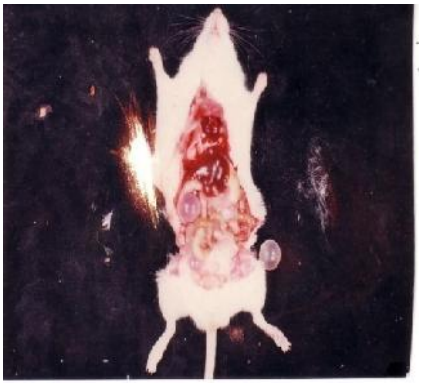

FIGURE 6: Gross appearance of secondary hydatid cysts in positive control group dissected 120 d p i (→)

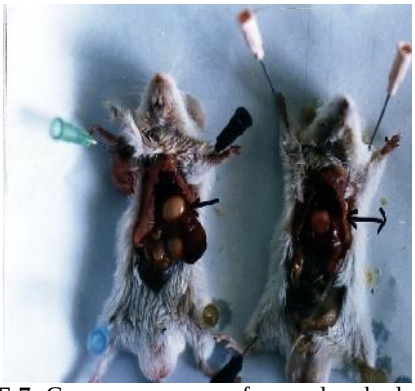

FIGURE 7: Gross appearance of secondary hydatid cysts in mice immunized with B-antigen

DISCUSSION

Genetic engineering applications always describe the use of pure DNA. Hydatid cyst ought to be intact so that the chance of contamination from a host tissue is less, Lung hydatid cyst, are excised intact, on the contrary to liver cyst however they are equally fertile, isolates were used for DNA extraction. The chromosomal DNA yield was satisfactory pure and the concentration was high, matching that obtained in [9]. An approach for cloning of hydatid cyst DNA in bacteria was achieved by a previous study[9], recombinant DNA yield was adequate in concentration and purity to be used in subsequent experiments. Applying a new antigen in immunization procedures required a comparison with an already well-established antigen such as native B antigen. Liver hydatid fluid of the human is thought to be a good source of highly antigenic substances, contains more proteins than psc from any of other intermediate hosts. Human hydatid fluid contains 17 to over 200 mg of protein per 100 ml, the source of such proteins being from the psc as well as host proteins [20].This was the reason for preparing antigen, from human liver cysts in the present study, the enriched fraction of B-antigens gave the highest sensitivity in serodiagnosis of cystic echinococcosis in their ability to detect IgG antibodies in sera of naturally infected sheep [21] . DDT test was used to detect affinity between antigens and antibody through formation of a precipitate line in the area of reaction due to their diffusion toward each other in semi solid gel[22]. IHA being highly efficient in detecting immune response to hydatid antigen[24]. Recombinant plasmid DNA was the most efficient antigens in eliciting antibodies in high titer in both im and sc groups using 0.3ml this in agrement with[25] using recombinant EG95 antigen effectively primed the humoral response, as

judged by high IgG anti-EG95 titer detected two week after a booster dose. Being small in size recombinant plasmid DNA is better introduced inside the cells, [4] mentioned that DNA appears either integrated into the cell or to be maintained for long periods in an episomal form to be expressed into its product proteins which in turn are to be assembled on antigen presenting cell surface to be recognized by T lymphocytes. These might have taken apart in this job in our experiment since part of DNA was introduced by sc route. While chromosomal DNA is large in size encompassing all the genome of millions psc so that it will be difficult to be introduced inside the cells , however, part of it succeeded in the process and elicited immune response as indicated from raising antibody titer. The use of calcium chloride augmented the uptake of DNA by the cells; it's known to change the permeability of the cell membrane for different DNA molecules used in molecular cloning and in bacterial transformation[26]. Native B antigen, was also efficient in inducing raised antibody titer, indicating that antigens are easier to be taken up by antigen presenting cells .Antigens extracts from fluid, psc and its extraction / secretion can induced antibodies at five weeks post immunization [27] which is in agreement with our results. All the preceding findings mentioned plus the good health of animals derived the decision on using the dose of 0.3 ml in the program of vaccination planned for the experimental work later on. Animals receiving different types of vaccine exhibited a clear decrease in the mean number of hydatid cysts, The lack of natural and acquired immunity in the positive control group was responsible for the 100% infection. Recombinant plasmid DNA was the best of all in the induction of protection against challenge. This result matched that of [28] using recombinant vaccine (GE95) against challenge infection with *E. granuolsus* eggs induced a high immune response in sheep reaching 100%. The results was higher than[29], the reduction was ranged from 79-80% against oral challenge with *E-granulosus* eggs. The use of chromosomal DNA antigen was also highly efficient in reducing the number of developing cysts at 120 dpi. These results, was more than the result of [30] who obtained less than 80% protection. Chromosomal DNA contains a good part of the genome of hydatid cyst that expressed into many antigenic proteins that initiated an immune response more than a single antigen (antigen B) could elicit[31]. (5&6) mentioned that DNA vaccines delivered by injection, put genes into some cells muscle or langerhans lead to their uptake by cells in the vicinity of the inserted needle. Once inside cells, some of the vaccine make their way to the nucleus and instruct the cell to synthesize encoded antigenic proteins leading to both a humoral response and CMI. The encoded protein is expressed in the host in its natural form. There is no denaturation or modification and the immune response will be directed to the antigens exactly as it is expressed by the pathogen[32]. DNA vaccines also cause prolonged expression of the antigens, which generates significant immunological memory. Decline in the antibody titer was noticed in recombinant plasmid groups after 120 dpi. It was significantly ($P<0.05$) .The latex test could not detect a low concentration of antibodies produced in infected immunized groups. The concentration of soluble antigen used for coating latex particles and the suspending buffers influence the sensitivity of the test making it only moderately sensitive in detecting antibodies[24]. The findings suggest the presence of cell mediated immunity exemplified by the swelling in the footpad caused by inflammatory odema and infiltrate[33] referred to the subset of T-helper cells responsible for DTH responses, continue to be active throughout the infection.DTH is caused by the various cytokines secreted by the mononuclear cells series especially T-lymphocytes and macrophages which are activated when they come across the insulting antigens. With time, depletion in these cells namely T-lymphocytes occurs associated with a decrease in footpad thickness, due to the low antigen induced lymphocyte transformation indices. Vaccination with DNA provides long-lived source of the protein and prolonged expression of the antigen as effective means for elicited both antibody and CMI .

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