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EVALUATION OF THE IMMUNE RESPONSE TO *T. VERRUCOSUM* VACCINES

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ABSTRACT

Dermatophytosis is considered as one of the most important fungal skin disease affecting both humans and animals otherwise its importance as zoonotic disease. Treatment of ringworm in cattle is expensive and time consuming especially on herd level. Vaccination can be a giant step forward to prevent the occurrence of mycozoonosis between the affected animals with ringworm and susceptible man. Fifty skins scraping samples were collected from animals (cattle, sheep and calves) suffering from skin lesions suspected to be ringworm. Then these collected samples were subjected to mycological examination and identification. *T. verrucosum* is the main causative agent of collected skin scrapings. Two types of vaccines were prepared from this strain (Culture filtrate and formalin inactivated vaccines). Rabbit model was used for challenge testing of the vaccines. The rabbits were separated into four groups as follow: Each group contained 3 rabbits each one was injected subcutaneously with 1ml of culture filtrate with adjuvant twice for seven days intervals. Group No. 2 rabbits each one was injected intramuscularly with 1 mL formalin-inactivated with adjuvant. The 3rd group is control positive and 4th one is control negative. Vaccination programme is done via injection twice with one week interval and the challenge by scarification of the skin with 0.2 mL of 5×10^7 fungal elements/mL. For 3 days. Humeral immune response was assessed by measuring anti-*T. verrucosum* antibodies using ELISA technique (direct method). By using statistical analysis of ANOVA, the mean optical density of anti-*T. verrucosum* specific IgG level in rabbits artificially immunized with culture filtrate with adjuvant vaccine significantly increase from (1.97) post first vaccination to reach (2.43) post second vaccination and still high after challenge with the virulent *T. verrucosum* strain (2.32) when compared with control positive (1.57). The results revealed that both type of vaccines induce good humeral immune response in vaccinated animals. Culture filtrate vaccine used as a treatment in infected farm animals by two doses within 2 weeks and noted that healing occurred.

Keywords: Verrucosum Vaccine, Dermatophytosis, Immune Response, Zoonotic Disease

1. INTRODUCTION

Dermatophytosis is common and clinically multifaceted fungal skin disease affecting both humans and animals and causes more economic losses among livestock. There is definitely a need for effective prophylaxis against the disease as hygienic and preventive measures often fail Gudding and Lund (1995). Vaccination can be a good method to prevent the occurrence of mycozoonosis between the affected animals with ringworm and susceptible man particularly in regions having predisposing factors such as close contact with the animal, traditional farming and cold winter season (Ozkanlar *et al*., 2009). The first trial for immunotherapy against dermatophytosis was recorded in Russia. They applied a dermatophyte-killed vaccine prepared from six dermatophyte species for treatment of infected animals. The treated animals showed a higher rate

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of recovery Fomin and Rozumnyj (1967). For many years ago the concept of developing vaccines against fungal diseases attracted little interest but this was changed in the past fifteen years because the dramatic increase in the incidence rates of fungal diseases worldwide (Cutler *et al*., 2007). So the present study was planned to cover the following: Isolation and Identification of the main causative agent of dermatophytosis in cattle onto mycological media, Preparation of different types of vaccines from the main isolated common fungi, Measurements of immune response developed against the prepared fungal vaccines and Determination of the immunotherapeutic value of the prepared vaccines in field cases.

2. MATERIALS AND METHODS

2.1. Specimens

Sample collected for isolation of dermatophytes according to (Sinski *et al*., 1979). About fifty specimens of (Hairs and skin scraping) were collected from affected calves (35), adult cattle (10) and sheep (5) suffering from ringworm lesions from different areas.

2.2. Isolation and Identification

Mycological examination of *T. verrucosum* by direct microscopic examination of broken hairs and some of skin scarping of collected samples by 20% KOH method. Then examined for fungal elements hyphae and spores around (ectothrix) or within the hairs (endothrix). Isolation of *T. verrucosum* by inoculation of specimens from different diseased animals onto Sabouraud`s dextrose agar with antibiotics (Chloramphenicol) and actidion and enriched with thiamine and inositol, the inoculated plates were incubated at 25 and 37°C for 3-4 weeks. The isolated fungi were identified according to macroscopical and microscopical morphology of the isolates to identify of micro and macro conidia of *T. Verrucosum*.

2.3. Preparation of Vaccines

2.3.1. Preparation of Culture Filtrate Vaccine

Selection of *T. verrucosum* strain used for vaccines preparation must be according to criteria reported by (Brandebusemeyer, 1990). Culture filtrate vaccine prepared according to (Gudding and Naess, 1986; De Boer and Moriello, 1993). Briefly selected isolate of *T. verrucosum* was inoculated into 500 mL Erlenmeyer flasks containing 300 mL of Sabouraud`s dextrose broth enriched with thiamine and inositol and incubated at 37°C for 4-6 weeks. The submerged growth of *T. verrucosum* were harvested in sterile beaker and stored at 4°C. The culture filtrate was also harvested and filtrated then stored at -20°C. At using mixing culture filtrate with adjuvant (Montanide) by equal amount.

2.3.2. Preparation of Formalin Inactivated Vaccine

Preparation of formalin inactivated vaccine according to (Rybnikar *et al*., 1986; De Boer and Moriello, 1993). Briefly formalin inactivated was prepared from (conidia and mycelia of fungus). The selected isolate of *T. verrucosum* was cultured in Sabouraud`s dextrose agar enriched with thiamine and inositol and incubated at 37°C for 15-21 days. Then the grown fungus was harvested into sterile bottles. The culture was killed by addition of formalin 0.3% later homogenized. Then filter by sterile gauze or sterile Whitman filter paper to remove large particles, where culture suspension was adjusted to approximately 5×10^7 fungal elements/mL. Then tested the purity and sterility of vaccine, at using adding of suitable carrier (montanide adjuvant) by equal amount.

2.4. Preparation of *T. Verrucosum* Antigen

Preparation of *T. verrucosum* antigen for evaluation of the immune responses. According to (Voller and Bidwell, 1986) through two steps, lyophilisation and grinding of *T. verrucosum* culture. Briefly, fungal mats were harvested and distributed in sterile 100 mL glass beakers. Then added equal volume of the lyophilized medium (skimmed milk) was added in a manner that the whole mixture did not exceeded 1/3 of the beaker volume for effective lyophilisation. Lyophilisation process was then carried out for 48 h using lab conco lyophilizer (Lyph Lock 12, USA). The lyophilized dermatophytes cultures were ground under aseptic conditions for fine powder. Extract antigen was prepared according to (Wawrzkiewicz and Wawrzkiewicz, 1992). Powdered mycelia (1 g dry weight) was mixed with 50g glass beads (0.1-mm diameter) and 10 mL of 0.01 M PBS PH The mixture was processed for 2 min in a rotatory homogenizer cooled with a stream of liquid CO_2. The homogenized mixture was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was recovered, passed through 0.45 um Millipore filter, aliquot and stored at -20°C until used.

2.5. Experimental Groups and Protocol

Twelve rabbits of mixed breeds apparently health and tested for freeing from dermatophytosis weighted from 1 kg to 1.5 kg they feed on special rabbit feed and divided into 4 group, each of which contains 3 rabbits, the first group was given culture filtrate vaccine by 1 mL s/c twice with one week interval 2nd group was given formalin inactivated

vaccine also by 1 mL s/c twice with one week interval. 3rd group was considered as control positive which challenged only. The 4th group was considered as control negative which was neither vaccinated nor challenged only. The 1st, 2nd and 3rd groups were challenged by scarification of the skin with 0.2 mL of 5×10^7 fungal elements/mL for 3 days shown in **Table 1**. Collection of the serum for measurement of immune response against the inoculated vaccines with following protocol, prior vaccination (zero time), after first vaccination within 7 days, at 10 days after second vaccination and after challenge.

2.6. ELISA Tests

Serum samples were analyzed for determination of antibody levels using ELISA technique. Humeral immune response was assessed by measuring anti *T. verrucosum* antibodies using direct method of ELISA according to (Sparkes *et al*., 1995; Bagut *et al*., 2013).

3. RESULTS

3.1. Prevalence of *T. Verrucosum* Infection

Examination of 50 hairs samples collected from adult cattle (10), calves (35) and sheep (5) affected by skin lesions suggestive of dermatophytosis, (60%) animals proved to be positive. The results obtained in **Table 2 and Fig. 1** proved that calves showed higher isolation rate and yielded as from calves 23 (46%) isolates, followed by adult cattle, 6 (12%) isolates and sheep 1 (2%) isolates.

3.2. Isolation and Identification of *T. Verrucosum* Strains

In this study we identified *T. verrucosum* depending on macroscopic appearance. Where it was very slow growing with heaped up, button like with folded white colony and non-pigmented reverse side as shown in **Fig. 2** and on microscopic appearance, the fungus gave characteristic chlamydospores arranged in chains as shown in **Fig. 3** and using slide culture technique for demonstration of macro and micro conidia, where it give clavate to pyriform micro conidia as shown in **Fig. 4**.

3.3. Immune Response Developed Against Dermatophytes Vaccines

3.3.1. Humeral Immune Response Developed Against Culture Filtrate with Adjuvant Vaccine

Humeral immunity was assessed for evaluation of immune response against prepared vaccines through using ELISA technique. Mean optical density of representing antibodies titter of (anti-*T. verrucosum*) in sera of rabbits artificially immunized with culture filtrate antigens with adjuvant increase from (1.97) after first vaccination to reach (2.43) post second vaccination and mean optical density of anti-*T. verrucosum* specific IgG were significantly higher after challenge with virulent *T. verrucosum* strain reach to (2.32) in compared with control group (1.57) and the analysis showed that no significant differences between level of immunity after first vaccination and booster dose of vaccine. As shown in **Table 3 and Fig. 5**.

3.3.2. Humeral Immune Response Developed Against Formalin-Inactivated Vaccine with Adjuvant

The mean optical density of representing antibodies titter of (anti *T. verrucosum*) in sera of rabbits artificially immunized with formalin-inactivated vaccine with adjuvant increase from (1.7) after first vaccination to reach (2.28) after booster dose of vaccine and mean optical density of anti-*T. verrucosum* specific IgG were significantly higher reach to (2.30) after challenge with the virulent *T. verrucosum* strain in compared with control group (1.57) and the analysis shown that no significant differences between level of immunity after first vaccination and booster dose of vaccine as shown in **Table 4 and Fig. 6**.

3.3. Results of Challenge Test in Rabbits

Three groups of rabbits (vaccinated by culture filtrate, vaccinated by formalin-inactivated vaccine and control +ve group) were challenged at 10 days post second vaccination by virulent strain of *T. Verrucosum*. The result revealed that control +ve group showed alopecia with erythema and scales formation in site of infection and by mycological examination were positive for fungal elements (septated hyphae and arthrospores) using low and high power of microscopical examination, as shown in **Fig. 7**. While immunized groups of rabbits appear clinically normal within one week after challenge and within 3 weeks, the hair completely appear normal and were negative for mycological examination, as shown in **Fig. 8**.

3.4. Using of Culture Filtrate in Treatment of Dermatophytosis in Field Cases

The prepared culture filtrate of *T. verrucosum* were used for treatment of field cases suffering from dermatophytosis through injection of the culture filtrate.

Table 1. Experimental design and immunization of rabbit for *T. verrucosum* vaccines

Group	No. of animals	Types of vaccine	Dose and rout	Number of dose	Challenge (dose and rout)
1	3 rabbits	Culture filtrate formalin	1 mL s/c	twice within one week interval	Scarification of skin.
2	3 rabbits	-inactivated vaccine	1 mL i/m		0.2 mL of 5×10^7 fungal
3	3 rabbits	Control +ve	-	-	elements/mL for 3 days
4	3 rabbit	Control –ve	-	-	

Table 2. Total number of collected samples and prevalence of positive one

Type of samples	No. of collected samples	Affected animals species	Positive		Negative	
			No	(%)	No	(%)
Skin scraping and hairs	50	Cattle (10)	6	12	4	8
		Calf (35)	23	46	12	24
		Sheep (5)	1	2	4	8
Total number	30	60%	20	40%		

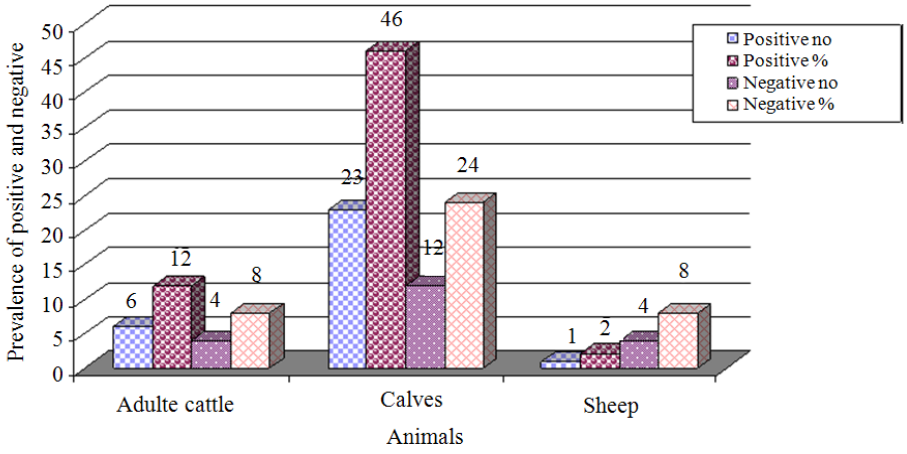

Fig. 1. Total number of collected samples and prevalence of positive one

Fig. 2. *T. verrucosum* on Sabouraud's dextrose agar

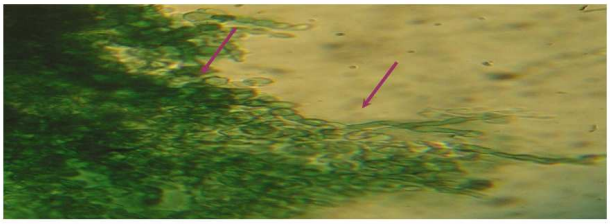

Fig. 3. Showing typical *T. verrucosum* chains of chlamydoconidia

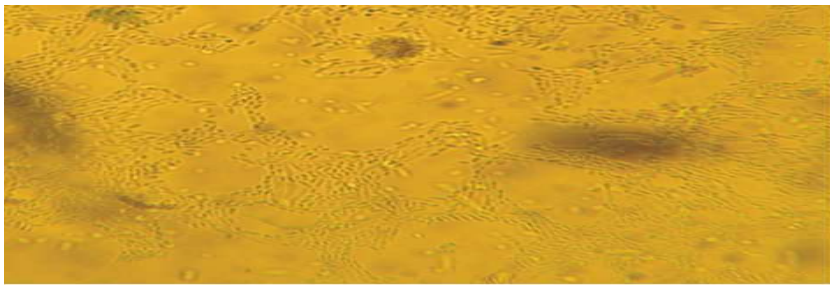

Fig. 4. Clavate to pyriform microconidia of *T. Verrucosum*

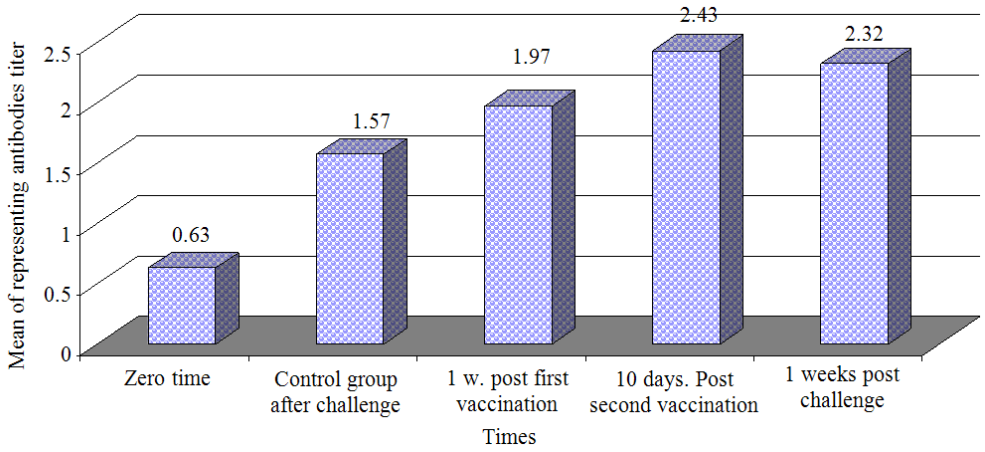

Fig. 5. ELISA representing antibodies titter of anti-*T. Verrucosum* IgG in sera of rabbits immunized with Culture filtrate with adjuvant

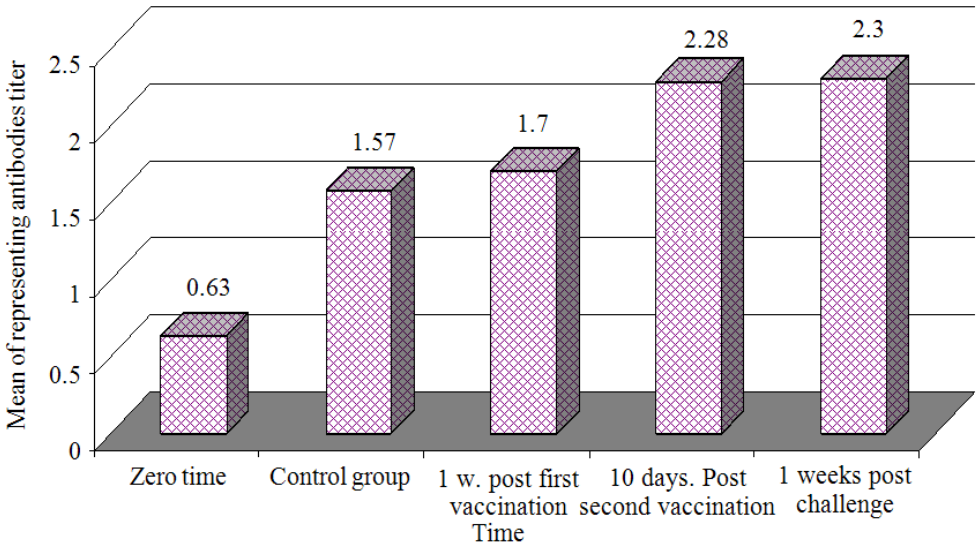

Fig. 6. ELISA representing antibodies titter of anti-*T. verrucosum* in sera of rabbits immunized with formalin-inactivated vaccine with adjuvant

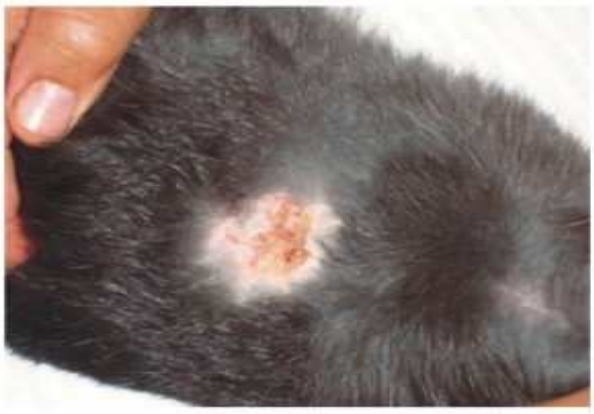

Fig. 7. Showing control +ve rabbit with alopecia and erythema and scales formation after infection with virulent strain of *T. verrucosum*

Fig. 8. Vaccinated rabbit after 3 weeks of challenge

Fig. 9. Showing Ear of cow with ringworm

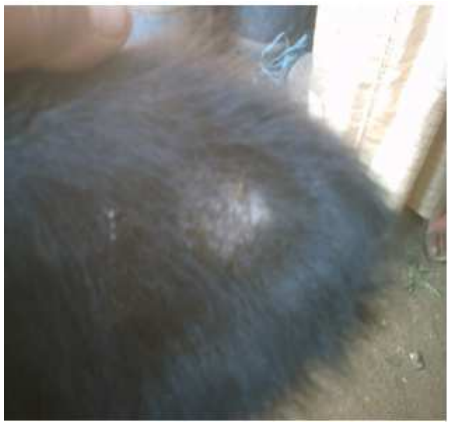

Fig. 10. After first dose of culture filtrate injection

Fig. 11. Seven days after second dose of culture filtrate

After injection of first dose of culture filtrate, the lesions began to heal and after the second dose within 7days, the diameter of lesions was noticed smaller and hair began to grow up and completely healing was noticed at 3 weeks after second dose. This shown in **Fig. 9-11**.

4. DISCUSSION

Dermatophytosis is one of the most frequent skin diseases of farm animals and consequently of human beings. Contagiousness among animal communities, high cost of treatment, economic losses, difficulties of control measures and its public health importance activated more concern with treatment and control even in trails of developing more vaccines against dermatophytosis. Within the present study, out of 50 skin scraping collected from cattle, calves and sheep with skin lesion, dermatophytes due to *Trichophyton verrucosum* were detected in 60% of examined samples. The highest rate of isolation was from calf samples 23 (46%) followed by cattle 6 (12%) and sheep 1 (2%) as shown in **Table 3 and Fig. 1**.

Table 3. ELISA mean of (anti-*Trichophyton verrucosum*) in sera of rabbits immunized with Culture filtrate with adjuvant

After challenge	Post second vaccination	Post first vaccination	Control group after challenge	(Zero time)	Samples
2.41	2.377	1.789	1.864	0.405	Samples1
2.43	2.494	1.902	1.774	0.91	Samples 2
2.142	2.435	2.223	1.093	0.586	Samples 3
2.32±0.9285[a]	2.43±0.3378[a]	1.97±0.12999[ab]	1.57±0.24339[b]	0.63±0.14772[c]	Mean and Std. Error

* Mean having the same titter are not significantly different (p>0.05). Different titers means significantly

Table 4. ELISA mean of (anti-*Trichophyton verrucosum*) in sera of rabbits immunized with formalin-inactivated vaccine with adjuvant

After challenge	Post second vaccination	Post first vaccination	Control group after challenge	(zero time)	Samples
2.422	2.08	1.663	1.864	0.405	Samples1
2.080	2.407	2.235	1.774	0.910	Samples 2
2.408	2.363	1.253	1.093	0.586	Samples 3
2.30±0.11174[a]	2.28±0.10246[a]	1.7±0.28476[ab]	1.57±0.24339[b]	0.63±0.14772[c]	Mean ± SE

*Mean having the same titter are not significantly different (p>0.05). Different titres means significantly

These result shown that dermatophytosis are usually more prevalent in young animals than old and this agree with Kamyszek (1975); Takatori *et al.* (1993) and Papini *et al.* (2008) concluded that calves of any sex, breed, age and of any of the farm condition considered are highly exposed to the risk of ringworm infection and this can be explained as young calves have incomplete development of immune system in addition to over crowdness and mal nutrition which act as stress factors on calves. *T. verrucosum* was identified on the basis of macroscopic and microscopic observations characterizing the colonial morphology (**Fig. 2 and 3**) and the presence of chlamydospores arranged in chains (**Fig. 4**) based in the identification key of the veterinary mycology laboratory manual Hungerford *et al.* (1999) and Similar findings were observed by Hassan (2002) and Calina *et al.* (2010) *T. verrucosum* needs more work concerning its control by developing more effective vaccines agree with Pier *et al.* (1993). Within the present study Two types of vaccines (culture filtrate and formalin-inactivated) were prepared. ELISA technique was used for estimation of humeral immune response against the prepared vaccines. Similar studies were performed by Almeida (2008) and Elad and Segal (1995) who assessed the humeral immune response in sera of calves immunized against *T. verrucosum* ringworm.The results presented in **Table 3** and **Fig. 5** illustrate that statistical analysis show that the mean optical density of anti-*T. verrucosum* antibodies in sera of rabbits experimentally immunized with culture filtrate vaccine with adjuvant. The increase recorded was (1.97) after the first vaccination and increase to reach (2.43) post second vaccinations. The mean optical density of anti-*T. verrucosum* antibodies was significantly higher (2.32) after challenge with virulent *T. verrucosum* isolate compared with the control +ve group (1.57). This agree with Gonzalez *et al.* (1997), Bratberg *et al.* (1999) and Pier *et al.* (1995) they found that culture filtrate of *T. verrucosum* have proteolytic enzymes and keratinolytic proteinase (KPase) enzymes, which was related to fungi biomass and found to be highest in Sabouraud's broth. These enzymes stimulate immune response. Concerning the results obtained in **Table 4 and Fig. 6**, the mean of optical density of anti-*T. verrucosum* antibodies in sera of rabbits immunized with formalin- inactivated vaccine showed increase from (1.7) post first vaccination to reach (2.28) after booster dose of the vaccine. The level of anti-*T. verrucosum* antibodies was higher (2.30) after challenge with the virulent strain of the organism compared with the control group (1.57). These results go hand in hand with those obtained by Grzywnowicz *et al.* (1989) and Lee *et al.* (1988). The efficacy of the prepared vaccines was tested through challenge three groups of rabbits (vaccinated by culture filtrate, vaccinated by formalin-inactivated vaccine and control +ve group) were challenged at 10 days post second vaccination by virulent strain of T. verrucosun The result revealed that control +ve group showed alopecia with erythema and scales formation in site of infection and by mycological examination were positive for fungal elements (septated hyphae and arthrospores), as shown in **Fig. 7**. While immunized groups of rabbits appear clinically normal within one week after challenge and within 3 weeks, the hair completely appear normal and were negative for mycological examination as shown in **Fig. 8**, these results found support from the results obtained by Heidy (1998). It is worth to mention

that culture filtrate vaccine of *T. verrucosum* prepared within the current study was used in a trial for treatment of field cases of animals suffering from ringworm lesions. This kind of manipulation gave good results in curing the disease through two doses at one week interval (**Fig. 9 and 10**).

5. CONCLUSION

The results revealed that both type of vaccines induce good humeral immune response in vaccinated animals. Culture filtrate vaccine used as a treatment in infected farm animals by two doses within 2 weeks and noted that healing occurred.

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