Vet.Med.J.,Giza. Vol.51, No.1. (2003):83-94.

EXPERIMENTAL EVALUATION OF DIFFERENT VACCINES AGAINST TRICHOPHYTOSIS IN GUINEA PIGS AND CALVES

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Received: 14. 11. 2002

Accepted: 12. 12. 2002

SUMMARY

Hairs and skin scales from 80 cattle with skin lesions, resembling ringworm were examined mycologically and *T. verrucosum* was the main common fungus isolated from 3 cases (3.75%).

Four types of vaccines were locally prepared, namely living attenuated, formalin-inactivated, phenol-inactivated and irradiated vaccines. The later vaccine was produced using a dose of 2KGy cobalt gamma irradiation, which was sufficient for complete inactivation of *T. verrucosum* and used for the preparation of the vaccine. Evaluation of the produced vaccines for purity and sterility was done. Aqueous whole dermatophyte extract antigen was prepared by mechanical disruption of dried mycelial powder of *T. verrucosum*. The ELISA optical density mean values of anti-*T. Verrucosum* IgG in sera of calves vaccinated with the four types of vaccines were determined. Marked positive delayed skin reactions were clearly observed in all vaccinated calves with different types of the prepared vaccines 48h after injection. Living attenuated and phenol inactivated vaccines proved to be the best inducers of allergic response in both calves and guinea pigs and the other two vaccines also sensitized guinea pigs with smaller degree.

On the basis of the average values of the macrophage migration inhibition test in guinea pigs, it indicated that the highest cellular immune response of guinea pigs after the first and second injection was due to the variant of the prepared vaccine and the reactions were more intensive following the first dose of the vaccines than after the second injection. Challenge tests in both immunized calves and guinea pigs were discussed in details.

INTRODUCTION

Bovine dermatophytosis causes economic losses due to hide damage and restrictions in showing and marketing infected cattle. Dermatophytosis caused by *T. verrucosum* is also a common zoonosis among farm workers. Control and eradication of this disease is therefore highly desirable (Gudding and Lund, 1995). Although ringworm is a superficial skin infection of cattle, it may have a significant impact on management in affected herds (Holubek, 2000). Once the disease is introduced into a herd, it spreads easily from one animal to another. The inflammation of the skin is associated with unthriftiness and general discomfort in affected cattle; also secondary bacterial infection may occur (Kielstein et al., 1998).

Treatment of clinical ringworm in cattle is expensive and time consuming. There is definitely a need for effective prophylaxis against the disease as hygienic and preventive measures often fail. (Gudding and Lund, 1995).

Modern control of bovine trichophytosis consists in administering different immunopreparations. Up till now some vaccines have been developed and/or applied in veterinary practice against ringworm (Wawrzkiewicz and Wawrzkiewicz, 1992). Immunization using live or killed fungi, their extracts, or their metabolic products has been now attempted (Rybnikar, et al., 1998).

Thus this work was planned to prepare different inactivated and living vaccines against T. verrucosum as well as the evaluation of the protective efficacy of the vaccines by detection of both humoral and cellular immune responses produced by different prepared vaccines.

MATERIAL AND METHODS

Collection of samples:

Hair and skin scrapings were collected from 80 cows showing suspected ringworm lesions on different parts of the body especially from head and neck. Specimens were collected in sterile Petri dishes during the period from Jan 2000 up to February 2001 and subjected to further mycological examination.

Laboratory examination of the collected samples:

A. Direct microscopical examination: according to (Sinski, 1974).

B. Isolation of dermatophytes:

Each sample was inoculated onto Sabouraud's dextrose agar (SDA), cycloheximide and an antibacterial antibiotic enriched with thiamin and inositol. One of the inoculated plates was incubated at 25°C and the other at 37°C, and kept under observation daily for up to 4 weeks. The suspected colonies were examined by Needle mount method and slide culture technique according to Collee et al. (1996).

Selection of dermatophyte strains used for local vaccine production:

Local isolates of *T. verrucosum* were selected according to the criteria, recorded by Brandebusemeyer (1990). living attenuated vaccine was prepared according to DeBoer and Moriello, (1993), formalin-inactivated vaccine and phenol inactivated vaccines were prepared according to Wawzkiewicz and Wawzkiewicz, (1992). Also Gamma irradiation inactivated vaccine was prepared by using gamma irradiation at dose rate of 0.406 Gy/ sec. (Rybnikar, 1994).

All prepared vaccines were tested for sterility and safety and given in 0.5ml twice 28 days apart for guinea pigs and 5ml I/M for calves.

Preparation of *T. verrucosum* antigen for evaluation of the immune responses:

Aqueous whole dermatophyte extract antigen was prepared according to DeBoer and Moriello (1993).

Experimental design:

A total of 15 calves were grouped into five categories. The first group comprised 3 calves vaccinated I/M with 5ml of the attenuated vaccine twice at 4 weeks interval, the second group vaccinated with formalin-inactivated vaccine, the third group vaccinated with Gamma-irradiated vaccine and the fourth group injected with phenol-inactivated vaccine in the same manner recorded in the first group. The fifth group was kept as un-vaccinated control group.

In addition a total of 75 adult albino guinea pigs were divided into 5 groups each of 15. The first vaccinated with attenuated vaccine, the second with formalin-inactivated vaccine, the third with gamma-irradiated vaccine, the fourth with phenol-inactivated vaccine. Each guinea pig received I/M a dose of 0.5ml of the used vaccine twice 4 weeks interval. The fifth group was kept as a control un-vaccinated group.

Two weeks after the booster dose of the vaccine, the immunizing potential and the protective efficacy of the tested vaccines were determined. Humoral immune response was assessed by measuring anti-dermatophyte specific IgG antibodies using ELISA as described by Voller and Bidwell, (1986). Measurement of cell-mediated immune response was done by trichophytin skin test in both guinea pigs and calves according to Wawrzkiewicz and Wawrzkiewicz, (1992) as well as migration inhibition test (MIT) in guinea pigs (Wawrzkiewicz and Wawrzkiewicz, 1992).

Challenge Test:

15-21 days-old virulent culture of *T. verrucosum* was adjusted to contain $5x10^7$ viable fungal elements/ml. An experimental infection of guinea

pigs was done 8 weeks after 1st vaccination for 2 consecutive days. 2ml of the prepared *T. verrucosum* strain suspension were applied to the scarified skin for 2 days. The inoculated animals were examined clinically one, 2, 3, 4, 5, 6 and 7, weeks post-infection and skin samples were taken for mycological examination.

Calves were experimentally infected 16 weeks after receiving the first dose of the vaccines. By rubbing with standardized suspension of the virulent strain of *T. verrucosum* for 3 successive days. The skin of calves was examined in the same manner as in guinea pigs.

RESULTS AND DISCUSSION

Ringworm is a common debilitating disease of calves, which is economically important to the breeders because it entails loss of production and cost of treatment. Animal trichophytosis is also an important problem for public health due to its easy transmission to man (Tirziu and Decun, 1999). In the present work, Trichophyton verrucosum isolates were the sole fungal isolates from examined specimens. Mycological examination of 80 cattle showing clinical skin affections revealed that 3 cases harboured *T. verrucosum* with and incidence of 3.75%. This result coincides with that observed by Munoz Cobenas et al. (1992) who recovered *T. verrucosum* only from outbreaks of dermatomycosis in cattle. The importance of protecting animals against dermatomycosis by immunization attracted the attentions of many authors. It was found that the radiocobalt dose of 1kGy did not produce complete inactivation of T. verrucosum. On the other side, a dose of 2kGy or more up to 5kGy proved to cause complete inactivation of *T. verrucosum*. In the present work, radiocobalt dose of 2kGy was successful in the production of the irradiated vaccine used in this work.

Four different types of vaccines were locally prepared namely: living attenuated, formalin-inactivated, radiated and phenol-inactivated vaccines. Evaluation of the produced vaccines for purity and sterility was done. In this study trials to evaluate the immune response of calves to the above mentioned vaccines were conducted by using ELISA.

Bratberg et al. (1999) developed an ELISA to measure antibody response in animals vaccinated against trichophytosis and found that ELISA to be superior to other tests in measuring serum antibody titers.

The results presented in Table (1) shows that the ELISA optical density mean values of anti-*T. verrucosum* IgG in sera of calves immunized with living attenuated vaccine reached its maximal level (0.376) at the fourth week post boostering, then

began to decrease gradually to reach 0.356; 0.336; 0.276 and 0.254 at 6th, 8th , 10th and 12th week post secondary vaccination. These optical density values were considered as functional background for the efficacy of this response. On the other hand, the values of anti-*T. verrucosum* IgG in the sera of calves immunized with formalin inactivated vaccine which reached its maximum after the 4th week post-primary vaccination (0.245) and after the 12th week post boostering (0.259) but the antibodies persisted for a long period of time. The sera of calves vaccinated with radiated vaccine showed an increase in its optical density that reached 0.395 at the fourth week post primary vaccination and at the second week post boostering, then fluctuating to reach again to its maximal level of 0.425 at the 10th week post boostering and persisted till the end of the experimentation. On the other hand, table (1) proved the protective efficacy of the phenol-inactivated vaccine in the

Table (1): ELISA mean statistical values of anti-*T.verrucosum* IgG in sera of calves immunized with different prepared vaccines.

Group of calves	Optical density values								
	Zero time	2 w. post 1st dose	4 w. post 1st dose	2 w. post 2nd dose	4 w. post 2nd dose	6 w. post 2nd dose	8 w. post 2nd dose	10 w. post 2nd dose	12 w. post 2nd dose
Group I Living attenuated	0.167	0.232	*0.349	**0.371	*0.376	*0.356	*0.336	0.276	0.254
	0.003	0.003	0.044	0.033	0.014	0.019	0.003	0.014	0.009
Group II Formalin-inactivated	0.1003	0.208	0.245	0.214	0.222	0.229	0.239	0.234	0.259
	0.027	0.004	0.006	0.014	0.023	0.019	0.008	0.028	0.033
Group III Radiated	0.123	0.310	0.395	**0.395	*0.385	*0.390	*0.38	*0.442	0.338
	0.037	0.033	0.016	0.023	0.013	0.031	0.003	0.033	0.000
Group IV Phenol-inactivated	0.130	0.278	0.351	0.387	**0.437	**0.457	**0.467	*0.432	0.298
	0.052	0.047	0.034	0.013	0.023	0.022	0.045	0.013	0.022

* Significant at p >0.05
** Significant at p > 0.01

Table (2): Trichophytin skin test in calves vaccinated with different types of the prepared vaccines.

Type of vaccine used	Serial No. of calves	Skin thickenss before injection (mm)	Skin thickness 48 h. post-injection (mm)	Difference in the skin thickness in response to Trichophytin (mm)		Difference in the skin thickness due to saline injection (mm)	
				Value	Average	Value	Average
Attenuated vaccine	1 2 3	8 7 7	35 17 40	27 10 33	23.33	1.5 1 1.5	1.33
Formalin-inactivated vaccine	4 5 6	6 13 12	22 25 22	16 12 10	12.66	1 1 1.5	1.16
Radiated vaccine	7 8 9	10 8 10	26 20 20	16 12 10	12.66	1.5 1 1	1.16
Phenol inactivated vcaccine	10 11 12	9 11 8	26 21 25	17 10 17	14.66	1.5 1 2	1.5
Control	13 14 15	11 12 7	12 14 9	1 2 2	1.66	1 1.5 1.5	1.33

immunization of calves against trichophytosis.

Table (1) illustrates comparison between the different vaccines used. After the 4th week post primary vaccination, the irradiated followed by phenol-inactivated, then living attenuated and finally formalin-inactivated vaccine showed highly protective efficacy in a descending manner with an optical density reached 0.395 ± 0.016; 0.351 ± 0.034; 0.349 ± 0.044 and 0.245 ± 0.006 respectively. These findings nearly coincide with of Rybnikar et al., (1998) and Tirzui and Decun (1999).

The problems of single vaccination of calves against trichophytosis have been discussed by some investigators (Gudding and Lund, 1995; Kielstein et al., and Rybnikar et al., 1998) but the protective effect of second prophylactic doses was quite evident compared with single dose only and the protective efficacy of single vaccination was not higher as compared with double inoculation.

Many authors reported the uses of delayed type hypersensitivity skin responses to intradermal injection of trichophytin antigen in calves infected or vaccinated with different *T. verrucosum* vaccines, with development of a specific immune response to dermatophytosis which have demonstrated the development of cell-mediated immune responses (Munoz Cobenas et al., 1992 and Tirziu

and Decun, 1999). In the present work, marked positive delayed skin reactions were clearly observed in all vaccinated calves with various types of vaccines at 48 hours post injection as shown in table (2). Attenuated living vaccine produced differences in the skin thickness varied from 10-33mm which was higher than both formalin-inactivated or irradiated vaccine which developed variation in the thickness between 10-16mm and slightly raised among calves vaccinated with phenol-inactivated vaccine with differences in the skin thickness in between 10-17mm. On the contrary, the control non-immunized calves showed insignificant differences in between 1-2mm with negative results. These findings coincide with the results obtained by Wawrzkiewicz and Wawrzkiewicz (1992).

As shown in table (3), the trichophytin skin test was also applied in groups of guinea pigs vacci-

Table (3): Trichophytin skin test in guinea pigs vaccinated with different types of the prepared vaccines.

Type of vaccine used	Skin thickness before infection (mm)	Skin thickness 48 hours post-injection (mm)	Difference in the skin thickness in response to Trichophytin (mm)		Difference in the skin thickness due to saline injection (mm)	
			Value	Average	Value	Average
Attenuated vaccine	3 2.5 2 4 2.5	8 5.5 4.5 8 5	5 3 2.5 4 2.5	3.4	0.5 0.3 0.3 0.4 0.5	0.4
Formalin-inactivated vaccine	3 2 2.5 2.5 2.5	5.5 3.5 5 4 4.5	2.5 1.5 2.5 1.5 2	2	0.4 0.3 0.5 0.3 0.5	0.4
Radiated vaccine	4 2 2 3 3	6.5 4.5 4.5 5 5.5	2.5 2.5 2.5 2 2.5	2.4	0.5 0.3 0.3 0.3 0.4	0.36
Phenol inactivated vaccine	4 2 2 2.5 3	7 6 4 5 6	3 4 2 2.5 3	2.9	0.5 0.5 0.4 0.6 0.5	0.5
Control group	2 4 3 2.5 2	2.5 5 3.5 3.5 2.5	0.5 1 0.5 1 0.5	0.7	0.5 0.4 0.3 0.3 0.4	0.38

Table (4): The effect of different kinds of vaccines on the degree of spleen cells migration inibition in guinea pigs:

		Type of vaccine			
		Living attenuated	Formalin-inactivated	Radiated group	Phenol-inactivated
	Day	Percentage of macrophage migration inhibition			
After the first vaccination	4th	21.1±0.2	4.1±0.31	8.4±0.30	20.5±0.36
	7th	29.2±0.4	10.1±0.34	18.6±0.53	28.3±0.41
	14th	61.0±0.39	42.3±0.30	[illegible]±0.33	63.0±0.41
	21st	59.1±0.3	25.6±0.59	50.0±0.43	59.6±0.28
	28th	16.0±0.59	5.9±0.36	4.7±0.34	11.3±0.59
After the second vaccination	4th	21.0±0.34	10.2±0.53	11.4±0.40	31.4±0.58
	7th	26.2±0.35	8.8±0.64	27.2±0.53	30.3±0.35
	14th	18.2±0.35	9.8±0.57	24.2±0.33	35.2±0.35
	21st	2.6±0.3	4.9±0.33	22.9±0.68	21.2±0.55
	28th	6.1±0.33	4.6±0.35	1.0±0.34	5.3±0.43
Control non-vaccinated group	-	7.9±0.34 0.1±0.28 3.4±0.58 5.2±0.46 5.1±0.43			

nated with the locally prepared vaccines. Living attenuated vaccine produced differences in the skin thickness varied from 2.5 to 5mm, then phenol-inactivated vaccine with differences in the skin thickness between 2-4mm. On the other hand, formalin-inactivated as well as the irradiated vaccines produced significant differences in the skin thickness in immunized guinea pigs but lower than both attenuated and phenol-inactivated vaccines. Vaccination of guinea pigs with T. verrucosum, vaccines elicits production of cellular immunity that can be measured by trichophytin skin test. These seem to agree with the observations recorded by Wawrzkiewicz and Wawrzkiewicz (1992); and Pier et al. (1995).

The general appraisal of the used vaccines on the basis of the averages obtained from the values of spleen macrophage migration inhibition test in guinea pigs is illustrated in table (4), it indicated that the highest cellular immune response in guinea pigs after the first and second injection were due to the variant of the prepared vaccines and it is apparent that the cellular immunological reactions were more intensive following the first dose of the vaccines than after their second injection.

Table (5) indicates that all calves vaccinated with living attenuated or phenol-inactivated vaccines required a resistance to virulent *T. verrucosum* as shown by challenge test and only slight superficial

Table (5): Results of challenge test in calves.

Type of vaccine used	Serial No. of calves	Clinical changes after challenge test per week						
		1	2	3	4	5	6	7
Attenuated vaccine	1	-	1+	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
Formalin-inactivated vaccine	4	-	1+	2+	2+	1+	1+	-
	5	-	1+	2+	2+	-	-	-
	6	-	1+	1+	2+	-	-	-
Radiated vaccine	7	1+	1+	2+	1+	-	-	-
	8	-	-	1+	2+	1+	-	-
	9	-	-	1+	1+	1+	-	-
Phenol inactivated vaccine	10	-	-	1+	-	-	-	-
	11	-	-	1+	1+	-	-	-
	12	-	-	-	-	-	-	-
Control non-vaccinated group	13	1+	2+	3+	4+	4+	3+	3+
	14	-	1+	3+	4+	4+	3+	2+
	15	-	1+	3+	3+	3+	2+	2+

- = No clinical changes
1+ = Single lesion
2+ = Changes up to 10 focuses
3+ = over 10 focuses
4+ = deep merging lesions

scaly skin changes that disappeared spontaneously within several days were noticed. Moreover, certain cutaneous changes did appear in the calves vaccinated with the formalin-inactivated or irradiated vaccines after being challenged, but all signs were temporary and disappeared later. These findings are nearly in agreement with that obtained by Wawrzkiewicz and Wawrzkiewicz (1992) who concluded that vaccinated calves exposed to challenge were to a large extent resistant to experimental infection with virulent strain of *T. verrucosum*. Moreover, Rybnikar et al. (1998) recorded that only 4.4% and 9.5% of calves challenged between days 14 and 25 after revaccination showed only slight mild clinical signs of ringworm and 99-100% were fully protected from day 28 and the immunity persisted for at least one year.

Table (6): Results of challenge test in guinea pigs.

Type of vaccine used	No. of guinea pigs	Clinical changes after challenge test per week						
		1	2	3	4	5	6	7
Attenuated vaccine	5	1+	1+	-	-	-	-	-
		2+	1+	-	-	-	-	-
		1+	-	-	-	-	-	-
		2+	-	-	-	-	-	-
		1+	-	-	-	-	-	-
Formalin-inactivated vaccine	5	3+	1+	2+	1+	1+	-	-
		2+	1+	2+	-	-	-	-
		3+	2+	1+	-	-	-	-
		1+	1+	-	1+	-	-	-
		2	1+	-	-	-	-	-
Radiated vaccine	5	2+	2+	-	-	-	-	-
		2+	2+	1+	-	-	-	-
		3+	1+	-	-	-	-	-
		1+	1+	1+	-	-	-	-
		1+	1+	1+	-	-	-	-
Phenol inactivated vaccine	5	1+	1+	-	-	-	-	-
		2+	1+	-	-	-	-	-
		2+	1+	1+	1+	-	-	-
		1+	1+	1+	-	-	-	-
		1+	1+	-	-	-	-	-
Control	5	1+	3+	4+	4+	4+	2+	2+
		1+	1+	2+	3+	3+	2+	2+
		1+	1+	3+	3+	3+	3+	2+
		1+	1+	2+	2+	2+	2+	2+
		1+	3+	4+	4+	4+	4+	3+

- = No clinical changes
1+ = Single lesion
2+ = Changes up to 10 focuses
3+ = over 10 focuses
4+ = deep merging lesions

As shown in table (6), it was observed that the majority of immunized guinea pigs with living attenuated or formalin-inactivated vaccine produced efficient protection against challenge and the lesions observed after challenge either in the form of slight crusty or cutaneous challenges not more than 10 focuses. Although the experimental infection with *T. verrucosum* resulted in the appearance of slight clinical lesions in immunized guinea pigs with formalin-inactivated or irradiated vaccines but the lesions were mild in comparison to the changes occurred in the control unvaccinated guinea pigs which showed trichophytic lesions from the beginning of the experiment till the end period (7th week). These findings nearly coincide with the observations recorded by Gudding and Lund (1995) and Rybnikar et al., (1998).

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