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Protection of Buffaloes Against Oedematous Skin Disease by Recombinent-bacterin and Toxoid-bacterin Vaccines

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Abstract: The protective efficacy of two formulated vaccines against *Corynebacterium pseudotuberculosis* biotype 2 was tested on male buffalo-calves (aged 6-8 months) from a herd free from Oedematous Skin Disease (OSD). Using a virulent strain of *C. pseudotuberculosis* biotype 2 (nitrate positive), locally isolated from buffalo severely infected with OSD, an experimental model of OSD was developed in which the manifestation of disease were equivalent to the naturally observed infection in Egypt. Subsequently the capacity of the two experimental vaccines to protect against experimental challenge was determined. The animals were divided into 3 groups each of 3 animals. Group 1 was immunized with a recombinant derivative of phospholipase D (r PLD) combined with formalin killed bacterin, while group 2 was immunized with crud supernatant of *C. pseudotuberculosis* toxoid and bacterin. Group 3 consisted of unvaccinated animals. All groups were challenged intradermally in hairless areas with live bacteria 6 weeks after last vaccination. Unvaccinated animals showed manifestations of OSD observed in naturally diseased animals. Following homologues experimental challenge, both vaccines were observed to confer protection against infection in all animals. In each of the 2 vaccinated groups there was a titer rise of anti-PLD antibodies following first dose of vaccination measured with ELISA test and titer decreased gradually irrespective of bosstering or challenge exposure. In second vaccinated group the titer of antibodies decreased gradually to reach the level of control unvaccinated animals through 3 months of challenge, while the titer in r PLD-bacterin vaccinated group titer persisted to 50 during the same period. The present investigation indicates that the development of OSD following intradermal inoculation with *C. pseudotuberculosis* can be reduced by an inactivated vaccine containing inactivated PLD in the form of recombinant PLD or formalin-inactivated crude supernatants in combination with whole formalin-killed bacterin. Vaccination is recommended to be performed at the end of February to cover the summer months before the appearance of *hippobosca equina* the main transmitter of the causative agent to buffaloes.

Key words: Corynebacterium pseudotuberculosis · Phospholipase D (PLD) · Oedematous Skin Disease (OSD) · bacterin · vaccination

INTRODUCTION

Oedematous Skin Disease (OSD) is an endemic disease of buffaloes in Egypt. It appears as outbreaks during the summer months, especially in Lower Egypt which is characterized by high humidity and high temperature. The outbreak form of OSD is usually associated with the breeding season of the blood sucking fly *hippobosca equine;* the main transmitter of the causative agent [1]. OSD is characterized by development of skin swellings which initially started from a hairless areas (sites of *hippobosca* infection) as axillary and groin regions then extends oedema to the hind or fore limbs, the belly and brisket region. These swell map are firm in consistency and usually involves the drainage lymph nodes which may enlarge to attain a size of watermelon. In affected cases, OSD may be associated with haematuria or respiratory distress and recumbancy and almost ends with death. Although mortality is low, morbidity is high and treatment of diseased animals may extend for months causing economic losses to farmers due to reduction of animal productivity; decrease in work efficiency of the

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animal, expensive medicaments and surgical intervention in some cases which represent a noxious situation for owners and veterinarians [2].

It is well documented that *C. pseudotuberculosis* biotype 2 (nitrate positive), exerts its pathogenesis by secretion of exotoxin (s) including phospholipase D (PLD) which has been implicated as the major virulence factor and incriminated as main player of pathogenesis of OSD [3, 4].

Both biotype 1 (nitrate negative) the causative agent of Caseous lymphadenitis (CLA) in sheep and goat and biotype 2 (nitrate positive) produced PLD, have different pathogenicity for guinea pigs, whereas, biotype 2 showed more rapid and reverse haemorrhagic lesions at site of inoculation associated with rapid death of the experimental animals as a result of the presence of other toxigenic factor(s) beside PLD that are produced by *C. pseudotuberculosis* biotype 2 [5].

Control of OSD presents several problems in part because there is insufficient knowledge of the epidemiology and pathogenesis of the disease. Treatment is often unsatisfactory since sever pathological changes may be present before clinical disease is recognized. Vaccination offers one approach to control of the disease on affected premises. The purposes of the work described here were development of suitable vaccine for protection against OSD; determination of the humoral immune response to vaccination or infection

MATERIALS AND METHODS

Nine male buffalo-calves (6-8 months) were obtained from a herd with no previous history of OSD and having no anti-PLD antibodies after examination of their blood with Enzyme Linked Immunosorbent Assay (ELISA).

Vaccines: Two vaccinal preparations were experimented.

Vaccine I: It was a combined vaccine composed of bacterin-toxoid components. Bacterin was prepared from a locally isolated strain of *C. pseudotuberculosis* biotype 2 obtained from severely infected buffalo with OSD and completely identified in National Research Center. It was named as Ramadan strain (name of the owner of buffalo). This strain was used for preparation of bacterin by the method of [6]. Bacteria were inoculated into brain heart broth contain 0.1% tween 80 and incubated at 37°C in shaker incubator for 48 hrs. The culture was pelleted by centrifugation and the pellets were washed 2 times with distilled water, once with 50% acetone, once will 100% acetone and twice with diethyl ether and air-dried. The dried pellets were weighed and resuspended in sterile saline solution containing 0.2% sorbitan mono-oleote(tween 80) and 0.1%o formalin and left to stand in room temperature over night. Killing of *Corynebacteria* and sterility were checked by standard culture techniques. Resuspension of pellets was performed in a percent so that each dose (4 ml) contained 2 mg of killed bacteria. The other toxoid component of the vaccine was prepared by cultivation of Ramadan strain on cooked meat medium (oxoid) by addition of 1.2 g/20 ml deionized water, then inoculated media were incubated at 37°C for 24 hrs, the contents of inoculated cooked meat medium were transferred to brain heart infusion broth containing 0.1% tween 80 and incubated for 48 hrs in shaker incubator at 37°C. After incubation the culture was left to stand at 4°C to precipitate coarse particles; then supernatants were centrifuged for further purification. The PLD contained in supernatants was assayed by SDS-PAGE of concentrated sample (20 times) and bands of PLD were detected by immunoblott technique using anti PLD hyper immune serum prepared in the BCSR. The PLD amounts were detected by analysis of the electrophorased gels using the computural Pro-Gel software in relation to the protection contents of electrophoresed supernatants previously measured by Lowry technique [7]. The synergistic hemolytic activity of the supernatants was determined by the method of [8]. The supernatants containing known amounts of PLD were inactivated by addition 0.1% formalin and left to stand in room temperature for overnight. Inactivation of supernatants (PLD) was checked by testing synergistic hemolytic activity and sterility by cultivation on brain heart agar. The inactivated supernatants (toxoids) were concentrated by lyophilization and weighed. The needed amounts of PLD were added to the killed bacterial suspension with continuous stirring so that each dose (4 ml) contain 500 µg PLD, after addition of PLD the mixture was stirred for additional 15 minutes.

Vaccine II: It composed of bacterin-mutated rPLD. The mutated recombinant briefly mutated PLD was prepared by inactivation of PLD through induction of mutation of *pld* gene by substitution of Histidine codon 20 by Tyrosine 20 and the mutated *pld* gene was expressed in *E.coli*. Bacterin prepared as previously mentioned. The mutated r PLD was added to bacterin suspension so that each dose (4 ml) contains 2 mg bacterin and 500 µg mutated r PLD.

Both vaccines were prepared by addition of the water phase (bacterin + toxoid) to the oil phase (Spanin mineral oil) in a percent of 3:7, respectively. The volume of the final vaccine dose was 4 ml that contains 2 mg bacterin plus 500ug PLD.

Vaccination and experimental challenge: Each vaccine was inoculated subcutaneously into a group of 3 animals distributed as 2 ml inoculated subcutaneously in the neck and 2 ml in the hind limb in one side of the animal. A third control group was inoculated with 4ml saline adjuvant mixture. Second dose of vaccine was administered 3 weeks after the first dose and challenge was performed 6 weeks after the last vaccination with 5 ml of 48 hrs brain heart infusion broth inoculated with Ramadan strain which contained 5 x 10^6 of living cells inoculated intradermally in hairless areas as axillary and groin folds.

Clinical observations: Temperature of all animals was recorded for 3 days after each inoculation and sites of inoculation and associated drainage lymph nodes were observed for appearance of any lesions.

Serology: Animal's immunological responses to vaccination and /or infection were determined by measurement of serum anti-PLD IgG by ELISA. Ninety-six well microtiter plates were coated with mutated r PLD. and bound anti-PLD IgG was detected by the use of anti-bovine conjugated with alkaline phosphatase (Sigma) which developed a color by addition of P-nitrophenyl phosphate substrate-calorimetric reactions were stopped and intensified reactions were stopped and intensified by addition of IN NaOH, 50 1/well and the absorbance of each sample at 450 mm was subsequently determined.

RESULTS

Optimization of infection model: In order to determine the optimal dose of *C. pseudotuberculosis* required to induce a similar disease manifestation in experimentally infected animals as observed in natural cases a, a dose titration was performed and proved to be 5 ml of 48 hrs brain heart infusion broth with tween 80 and contained 5 xlO^6 living bacterial cells/dose. The infection experiments to develop OSD must be performed during hot summer months as infection in winter months not resulted in typical lesions of OSD. All animals including 2 animals of the control group were challenged by intradermal inoculation of living *Corynebacteria* except one control-unvaccinated animal, which was inoculated intravenously with 2 ml of challenging suspension. But the animal died after 23 days

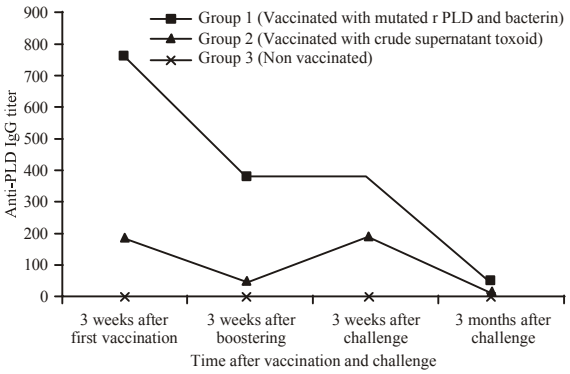

Fig. 1: Titers for antibodies against PLD

of challenge showing typical lesions of intoxication including lung, liver & kidney lesions and *C. pseudotuberculosis* was isolated from the infected organs. This result indicated that the suitable route for inducing experimental OSD during challenge experiments is through intradermal inoculation of challenging dose in hairless areas.

Vaccination and experimental challenge: Following vaccination, slight increase in temperature of vaccinated animals could be recorded, which subsides after 3 days. On the other hand, following challenge, temperature slightly rose in vaccinated animals and subsides after 3 days, while in unvaccinated control group, the rise of temperature prolonged for more than 3 days and gradually subsides after 7 days of challenge. No lesions of OSD could be observed at the site of inoculation, except for in the control unvaccinated group which showed typical lesions of OSD at sites of inoculation associated with enlargement of drainage lymph nodes, then swellings burst and discharge pus (Table 1)

Serology: humoral immune response was measured by ELISA using mutated r PLD as coating antigen. To compare immune response after first, second vaccination and post challenge, data are presented as average OD450 nm values in Table 2

Photographic picture 1 shows a typical lesion of oedematous skin disease in control animal after inoculation by a suspention of bacteria isolated from a local strain…..

To compare immune response among treatment groups data are presented as average OD450nm values per group and titers were determined as the reciprocal of the dilution that gave the maximal optical density.

Results in Table 3 and Fig. 1 show the highest titers of anti-PLD IgG in group 1(vaccinated with mutated rPLD

Table 1: Development of OSD at sites of inoculation invaccinated and control buffaloes

Type of vaccine	Animal No.	Lesions after 1st vaccination	Lesions After 2nd vaccination	Lesions after challenge
Recombinant PLD+bacterin	195	No lesion	No lesion	No lesion
	186	No lesion	No lesion	No lesion
	194	No lesion	No lesion	Slightly swelling
Formalin toxoid+bacterin	175	No lesion	No lesion	No lesion
	197	No lesion	No lesion	No lesion
	193	No lesion	No lesion	No lesion
Control non vaccinted	188	No lesion	No lesion	Large swelling that discharge pus
	199	No lesion	No lesion	Very large swelling extendedto the belly
	192	No lesion	No lesion	No lesion

Table 2: Mean antibody titration in buffaloes vaccinated with recombinant-bacterin and toxoid bacterin after first, second vaccination, 3weeks and 3months post challenge with *C.pseudotuberculosis*

1:600	1:800	1:400	1:200	1:100	1:50	VACCINE	Dose	Animal group
0.184	0.322	0.361	0.441	0.473	0.483	Recombinant+bacterine	Mean OD post first dose of vaccination	194-186-195
0.156	0.190	0.222	0.335	0.363	0.374	Toxoid+bacterin	Mean OD post firstdose of vaccination	171-197-193
0.135	0.148	0.156	0.172	0.197	0.218	Control	Mean OD post firstdose of vaccination	188-199-192
0.178	0.233	0.325	0.372	0.436	0.469	Recombinant+bacterine	Mean OD post seconddose of vaccination	194-186-195
0.177	0.184	0.210	0.241	0.276	0.316	Toxoid+bacterin	Mean OD post seconddose of vaccination	171-197-193
0.124	0.133	0.161	0.194	0.255	0.274	Control	Mean OD post seconddose of vaccination	188-199-192
0.237	0.246	0.311	0.391	0.527	0.586	Recombinant+bacterine	Mean OD 3 weekpost challenge	194-186-195
0.163	0.194	0.256	0.325	0.370	0.418	Toxoid+bacterin	Mean OD 3 week post challenge	171-197-193
0.184	0.222	0.234	0.303	0.355	0.364	Control	Mean OD 3 week post challenge	188-199-192
0.177	0.184	0.210	0.241	0.276	0.316	Recombinant+bacterine	Mean OD 3 month post challenge	194-186-195
0.144	0.161	0.165	0.194	0.240	0.253	Toxoid+bacterin	Mean OD 3 month post challenge	171-197-193
0.147	0.174	0.187	0.194	0.214	0.253	Control	Mean OD 3 month post challenge	188-199-192

Table 3: Titer of anti-PLD IgG in buffaloes vaccinated with recombinant -bacterin and toxoid-bacterin after first & second vaccination and 3 weeks & 3 months post challenge with *C. Pseudotuberculosis*

Group of animals	Titer after 1st vaccination	Titer after 2nd vaccination	Titer 3 weeks post challenge	Titer 3 months *post* challenge
194-186-195 (mutated rPLD + bacterin)	800	400	400	50
171- 197-193 (toxoid + bacterin)	200	50	200	Nil
188-199	Nil	Nil	Nil	Nil

Photo 1:

and bacterin), the maximum was obtained 3 weeks post first dose of vaccine and 3 weeks after booster dose the titer decreased and prolonged to 3 weeks after challenge and then the titer clearly decreased after 3 months of challenge. The titer of anti-r PLD IgG in general was lower than that in group "1" and reach its highest titration after the first vaccination and decreased 3 weeks after boostering, then increased during 3 weeks after challenge then decreased to the level of control non vaccinated group.

DISCUSSION

To our knowledge, this is the first study conducted to investigate the potential for vaccination against OSD in buffaloes in Egypt. It was report here the protection of buffaloed vaccinated with two different types of vaccines. The first combined vaccine was composed of inactivated PLD toxin prepared by genetic mutation in the active site of the *pld* gene encoding PLD exotoxin through substitution of Histidine 20 to Tyrosine 20 and the mutated *pld* gene was expressed in *E.coli* to express the mutated r PLD. The second component of the vaccine was the whole formalin-killed organism (bacterin) and was composed of toxoid prepared by formalin inactivation of the crud supernatant of *C. pseudotuberculosis* rich in PLD contents. The second component was the formalin killed whole *C. pseudotuberculosis* (bacterin). Both vaccines were adjuvated with span-mineral oil adjuvant. The dose of both vaccines was equal and contain 2 mg bacterin and 500jig of inactivated PLD. The route and dose of challenge with *C. pseudotuberculosis* were investigated in the present study (unpublished) and results proved that the suitable dose of challenge was 5 ml of brain heart infusion broth incubated at 37°C for 48 hrs and contained 5 x 10^6 of living *C. pseudotuberculosis* cells. The suitable route was the intradermal inoculation into the hairless areas as axillary and groin folds. Intravenous infection of *C. pseudotuberculosis* with the same dose resulted in death of animal through 23 days of inoculation without showing the typical lesions of OSD, but the animal showed manifestation of intoxication in the internal organs and *C. pseudotuberculosis* could be isolated from necrotic focci developed in the internal organ. Intradermal inoculation of buffaloes with *C. pseudotuberculosis* during the winter months (January and February) showed slight skin reactions and sites of inoculation, while inoculation in summer months inoculation of *C. pseudotuberculosis* resulted in appearance of typical manifestation of OSD. These results indicate that high temperature and humidity have relation to the development of OSD in summer months and that swellings of OSD is an interaction between the microorganism and host tissues which react in presence of certain temperature and humidity. Moreover, the appearance of OSD in summer months in the form of outbreaks was correlated to the information reported that summer months are the breeding season of the *hippobosca equina*, the main transmitter of the causative organism. Whole cells (bacterin) and toxoids were included in the vaccine in the light of the possibility that both the exotoxin and whole cell antigens may be important for development of immunity against *C. pseudotuberculosis* infection [9,10]. On the other hand, some authors reported that addition of bacterin to toxoid vaccines did not improve the protective potency of toxoid vaccine alone [11].

The protective capacity of both vaccines was demonstrated by the absence of skin reactions and OSD manifestation of the sites of challenge with virulent *C. pseudotuberculosis* if compared with unvaccinated control group which showed typical lesions of OSD. The present results showed that PLD inactivated either with formalin or without formalin by genetic inactivation of the *pld* gene could provide the same potency of protection. Although both vaccines showed equal vaccinal efficacy, yet they differ in stimulation of the immune system to produce anti-PLD IgG. The titers of antibodies developed after mutated PLD vaccination showed higher titers than with toxoid vaccines. This result agrees with observations of [9] who reported that native PLD is more immunogenic than toxoid form. Results show that in both vaccinated groups, the titer of antibodies was peaked after 3 weeks after vaccination and declined 3 weeks after boostering. Three weeks after challenge no increase in the titer in the first vaccinated group was noticed and gradually decreased after 3 months of challenge. But in group 2 the titer increased after challenge and gradually decreased to control level after 3 months of challenge.

The results in the present investigation showed that combined vaccines protected buffaloes from experimental challenge, but the period of protection provided by vaccines is not studied here, which needs further investigation especially the observation here which showed that anti-PLD IgG were declined to minimum after 3 months of challenge.

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