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Preparation and Field Evaluation of *Mycoplasma bovis*Membrane Vaccine in Egypt

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Abstract: ***Aim:To prepareM. bovis membrane vaccine and its evaluation in laboratory experiment and field study. Material and Methods:In this study, a laboratory experiment was carried out to evaluate the safety and potency of the prepared M. bovis vaccine. A field study was applied on three bovine dairy farms at Fayoum and Behera Governorates for 6 months. Detection of M. bovis antibodies was measured using indirect ELISA. Results:The laboratory experiment cleared that vaccinated& challenged group of rabbits with virulent field strain showed high immune response and the vaccine was able to protect rabbits against M. bovis infection. In the field study, a high immune response was detected in all vaccinated animals. Culture and PCR were negative in all vaccinated animals indicating that the vaccine provides a good protection against Mycoplasma bovis infection, while the contact animals gave positive results for isolation and PCR.Conclusion: M. bovis membrane vaccine was prepared from local field isolate. In the laboratory experiment vaccinated rabbits were protected against challenge with M. bovis virulent field strain. In the field study, the vaccinated cows produced high immune response with negative culture and PCR, indicating that the prepared vaccine was able to protect the animals against M. bovis infection.***

Keywords: *Mycoplasma bovis*, Membrane vaccine, dairy cows, indirect ELISA, PCR

1. Introduction

M. bovis is significant cause of sporadic outbreaks of clinical mastitis in small and medium size dairy herds and is more commonly identified as the etiological agent of recurrent cases of clinical and subclinical mastitis

Mycoplasmal infection causingbovine mastitis is increasing inprevalence and geographic distribution,and is of increasing concern tothe dairy industry [1]and[2].*Mycoplasma bovis*, which causes the most commonand most severe form of mycoplasmalmastitis, has been isolated frommastitic udders and bulk tanks duringherd outbreaks in many dairy producingareas throughout the world [3].In Egypt *M. bovis* was isolated from clinical, subclinical and tank milk of bovine dairy herds at different Governorates [4],[5] and [6].Since there is no effective antimicrobialtherapy or mass screening method for detecting infected animals and / orherds, prophylactic vaccination couldprove to be useful in controlling *M.bovis* mastitis [3] and [7].

Vaccination is a potential strategy to control *M. bovis* infection but efforts to develop efficacious vaccines against*M. bovis* for using in cattle. *M. bovis* have afforded some protection from respiratory disease in field trials[8] and [9]. [10]tested a commercial *M. bovis* bacterin in two herds of pre-weaned dairy calves with endemic *M. bovis* disease. The vaccine was not efficacious in the prevention of either *M. bovis* associated respiratory disease or otitis media. Other investigation was done by [11], they have reported protection of calves from mycoplasma respiratory disease after subcutaneous vaccination with killed whole cell bacterin.

Therefore, the present work aimed to control Mycoplasma mastitis infection in bovine dairy herds using *M. bovis* membrane vaccine.

2. Material and Methods

Identification of *Mycoplasma bovis* field isolate:

Culture media: PPLO Media was used according to [12]
Field isolate wasisolated from buffalo and identified by PCR, sequenced and submitted to Genbank (Egy-Bu-6-DK-12) with Accession No.JQ922410, was used for preparation of the vaccine

Polymerase Chain Reaction (PCR):

DNA extraction: The DNA extraction was carried out using QIA Amp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Primer selection: One set of primer encoding 16S rRNA gene was used for the detection of *M. bovis* as described by [13].The primer was prepared by Macrogen, Company, South Korea.
MboF: 5' – CCT TTT AGA TTG GGA TAG CGG ATG – 3'
MboR: 5' – CCG TCA AAG TAG CAT CAT TTC CTA T – 3'

PCR procedure: was performed on a Bio-Radcycler (S1000 Thermal cycler, USA).The reaction mixture (total volume 50µl) was 25 µl dream green PCR Master Mix (2X), Fermentas Company Cat. no. K1080, USA, 3µl target DNA, 1µl of each primer (10 pmol)and mixture was completed to 50 µl by nuclease free water.Amplification was performed by heating the sample at 94°C for 45 sec., 55°C for 1 min. and 72°C for 2 min. The amplification was performed for 35 cycles with final extension step at 72°C for 3 min. The PCR products were analyzed by agarose gel (1.5%) electrophoresis and visualized by staining with ethidium bromide and photographed.

Vaccine preparation:according to [14]

Preparation of antigen:*M. bovis* (Egy Bu6-Dak-12) was grown in PPLO medium for 48 hours and harvested by centrifugation at 14000 r.p.m. for 20 minutes. Pellet was washed for three times with phosphate buffer saline (PBS) pH 7.2. The pellet is re-suspended inPBS and the protein concentration was estimated as described by [15]

ProteoJET Membrane Protein Extraction Kit : Fermentas, Cat.no. K0321,EU was used according to the manufacturer

Vaccine preparation: according to [14].

Vaccine evaluation:
Determination of Hydrophilic-Lipophilic Balance (HLB) value of the oil emulsion: -[16] and [17].

a-Physical evaluation

- Emulsion stability test: according to Specification **WHO/M/13.R4** [18]
- Viscosity testing according to [19].
- Each 100 ml of vaccine containing 9.7 ml span 80 (Sigma), 59 ml paraffin oil (Sigma) as oil phase and 1.8 ml tween 80 (sigma) and 29.5 ml PBS (1mg/ml membrane protein)as aqueous phase.

b-Sterility testing (according to the Code of Federal Regulations "9 CFR"): For detection of Bacteria, Fungi and Mycoplasma contamination.

c-Safety and Potency test: Challenge test according to [11]:

Design of the laboratory experiment:

Four groups of New Zealand rabbits (9-10 weeks old) were weightingabout 1.5 kg were housed separately and vaccinated 0.5 ml S/C. These groups represented as:

1) Group A (vaccinated/challenged): 4 rabbits were inoculated with membrane vaccine then boostering after 2 weeks and challenged 2 weeks later with 0.2 ml(10^8cfu / ml) S/Cof field *M. bovis*(Egy-Bu-6-DK-12) .
2) Group B (vaccinated / not challenged): 4 rabbits were vaccinated2 weeks later S/C with 0.5 ml*M. bovis* Vaccine then boostering after 2 weeks and not challenged. These were monitored for adverse effects (Safety test) and antibody response.
3) Group C (challenged): 4 rabbits were inoculated with 0.2 ml of *M. bovis* field isolate S/Cafter 2 weeks.
4) Group D (unvaccinated / not challenged): 4 rabbits as control negative group.

The membrane immune response in the vaccinated groups was detected using ELISA (Mycoplasma bovis ELISA kit, Bio-X Diagnostics, Belgium)

Field study and sample collection: Milk samples were collected every two weeks from all vaccinated and contact animals, which were subjected to ELISA, culture and PCR.

3. Results

3.1 Laboratory Experiment

For evaluation the potency and safety of the prepared membrane *M. bovis* vaccine, a laboratory experiment was carried out in rabbits. Group (A) showed the highest antibodies titer after challenge and extended to the end of the experiment, followed by groups B and C. While the control negative (group D) was the least.

Concerning culture and PCR, group C gave positive results one week after challenge and extended to the end of the experiment. While groups A,B and D were negative for culture and PCR.(Fig.1).

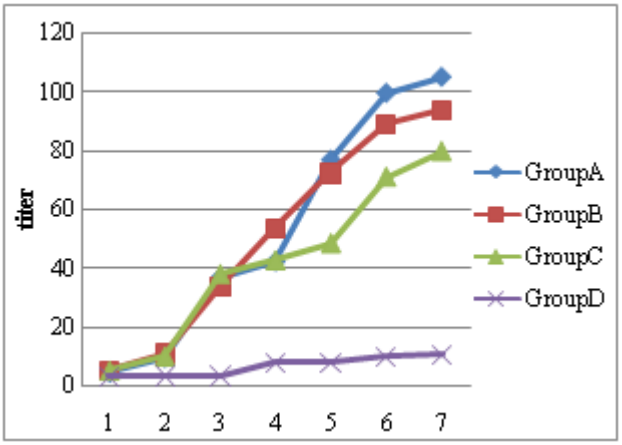

Figure 1: ELISA results of *M. bovis* membrane vaccine in rabbits

3.2 Field evaluation of the prepared *M. bovis* membrane vaccine

Two localities in Fayoum Governorate (farm1represented by 56 animals, farm2 represented by 80 animals) three herds were chosen from each farm, and in Behera Governorate, one farmrepresented by one herd (32 animals) was chosen. All animals were tested for the detection of *Mycoplasma bovis* infection using ELISA, isolation, and PCR.

The animals proved to be negative for all used tests were chosen for vaccination. The dose was 4 ml injected S/C, followed by booster dose after two weeks. Milk samples were collected every two weeks for six months from all vaccinated and contact animals were subjected to ELISA, culture and PCR. At Fayoum (Fayoum1&2) ELISA geometric mean titer started with (13.4-18.9) after two weeks post vaccination and increased to reach (153.24-195.2) at the end of experiment (24 weeks).While at Behera GMT was (23.56) after two weeks and increased to be (145.69)Table(1)

3.3 Isolation and PCR results of vaccinated herds:

The collected milk samples of the vaccinated herds were negative for isolation and PCRcontinued to give negative results till the end of the experiment, whereas the contact animals were positive for *Mycoplasma bovis* infection by isolation and PCR, indicating that the immune response due

to vaccination (vaccinated cattle were protected against the infection), Photo (1).

Table 1: Geometric mean titer of the herds from Fayoum and Behera Governorates vaccinated with local *M. bovis* vaccine

Governorate	*Fayoum Governorate*						*Behera*
Weeks	*Farm1*			*Farm2*			*Farm1*
	Herd1	*Herd2*	*Herd3*	*Herd1*	*Herd2*	*Herd3*	*Herd1*
2	14.98	14.98	13.4	18.92	15.03	18.5	23.56
4	46.34	38.9	44.19	22.2	25.32	27.2	32.64
6	62.11	58.49	53.29	26.5	30.03	44.4	45.66
8	71.93	62.11	77.14	36.66	36.17	50.15	52.51
10	83.9	71.93	95.58	50.4	49.21	59.21	64.66
12	105.84	105.84	115	70.66	68.37	61.73	75.68
14	110.66	110.66	130	75.34	72.15	72.74	98.20
16	113.27	115.27	134.5	80.53	81.65	83.61	95.56
18	132.45	133.29	160.21	105.23	113.32	105.2	107.71
20	164.3	165.2	175.2	132.1	123.52	121.5	119.36
22	180.85	171.32	190.42	140.1	139.2	135.35	128.35
24	189.27	175.2	195.2	155.21	162.47	153.24	145.69

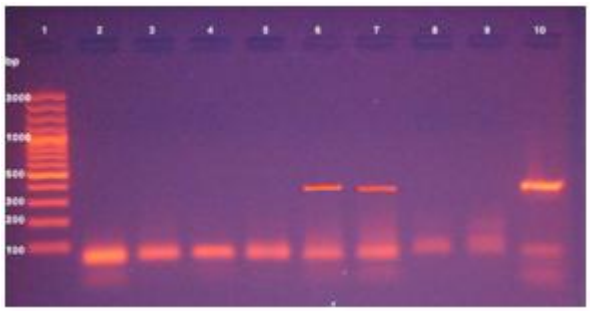

Photo 1: PCR results of vaccinated and non-vaccinated infected cows with Mycoplasma bovis mastitis
Lane 1: 100 bp DNA ladder Lanes 2, 3, 4,5and 8: vaccinated cows
Lanes 6 and 7: non-vaccinated infected cows with *M. bovis* mastitis (contact)
Lane 9: control negative Lane 10: control positive

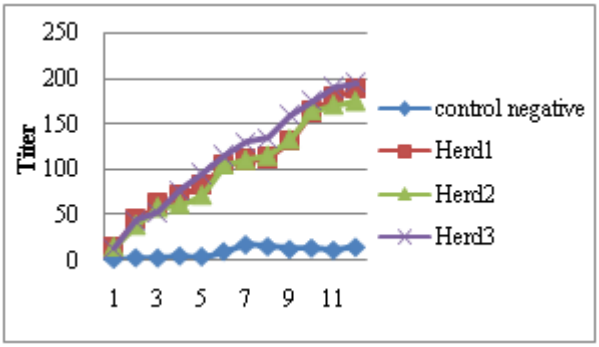

Figure 2: ELISA results of three herds at Fayoum Governorate (Farm 1) vaccinated with *M. bovis* vaccine

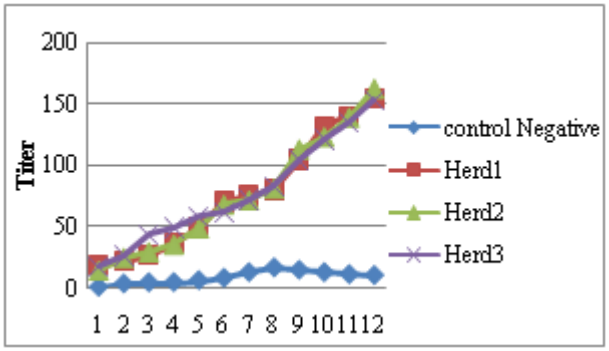

Figure 3: ELISA results of three herds at Fayoum Governorate (Farm 2) vaccinated with *M. bovis* vaccine

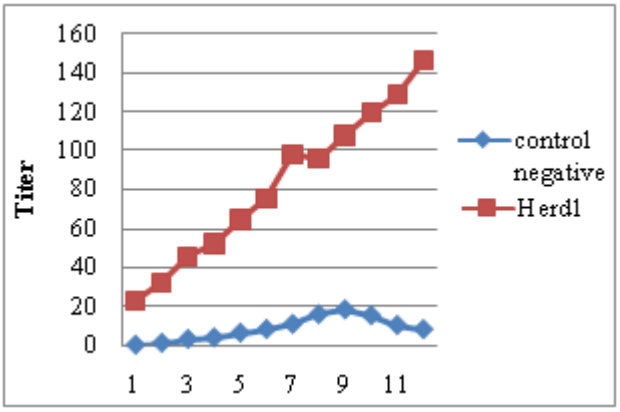

Figure 4: ELISA results of one herd at Behera Governorate vaccinated with *M. bovis* vaccine

4. Discussion

Mycoplasma bovine mastitis is a highly contagiousdisease that results in milk loss and culling of infectedanimals. Several species of *Mycoplasma* have beenassociated with mastitis (*M. bovis, M. californicumM.canadense, M. bovigenitalium, M. alkalescensM. arginini, M. bovihirnis* and *M. dispar*)[20].

In Egypt *M. bovis* was isolated from clinical, subclinical and tank milk of bovine dairy herds at different Governorates [4], [5]and [6]*M. bovis* strain isolated from clinical mastitis cases in buffaloes at Dakahlia Governorate identified using PCR, sequenced and submitted to GenBank. This isolate was used for preparation of *M. bovis* membrane vaccine which tested for safety and potency in rabbits showed the highest immune response detected by ELISA in Group A (vaccinated), followed by group B (vaccinated / challenged) and group C (challenged). These results agreed with [21]who mentioned that rabbits vaccinated with *M. bovis* and *M. bovigenitalium*saponized vaccine had the highest antibody titers. Also 10 concluded that calves vaccinated s/c with *M. bovis* bacterin and received two boosters at three week intervals showed lower respiratory disease (16.3%) when compared with control calves (27%).

In the present work, the prepared vaccine was evaluated in field study on bovine dairy herds in two governorates (Fayoum and Behera). Milk samples were collected from vaccinated and contact animals every two weeks and continued for 6 months.

M. bovis specific antibody levels detected by the indirect ELISA gradually increased after the booster dose to reach (198.2) in herd 3 (Farm1, Fayoum), while herds 2 (Farm1, Fayoum) gave a GM titer of (162.47), followed by the dairy farm at Behera Governorate (145.69) after 6 months. Culture and PCR were negative in the all vaccinated animals till the end of study. Also milk production was normal in quantity and quality without any pathological changes. On the other hand, the contact animals (infected with *M. bovis* mastitis) were positive for culture and PCR with pathological change in milk (secretion containing visible particles, seropurulent or aqueous suspension) and drop of milk yield.

[22] mentioned that vaccination with killed *M. bovis* systemically and in the mammary gland elicited local and systemic antibody. [23]indicated that sapon in combined vaccine can produce a specific humeral immune response to *M. agalactiae* over 6 months. Also [24] detected a high immune status with GMT 40.3, 3 weeks post-vaccination and has risen to 80.6 after 4 week and was maintained to 64 at the end of experiment.

Immunization of animals with high quality vaccines is the primary means of control for many animal diseases. In other cases, vaccines are used in conjunction with national disease control or eradication programs.

5. Conclusion

The present work aimed to prepare and evaluate *M. bovis* membrane vaccine prepared from local field isolate. Three farms at Fayoum and Behera Governorates were choice for the field study which extended to six months. The vaccine proves to be safe, efficacious and immunogenic.

6. Acknowledgment

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