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

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**Abstract:** Aim: To prepare *M. 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 a 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.

Mycoplasma infection causing bovine mastitis is increasing in prevalence and geographic distribution, and is of increasing concern to the dairy industry [1] and [2]. *Mycoplasma bovis*, which causes the most common, and most severe form of mycoplasmal mastitis, has been isolated from mastitic udders and bulk tanks during herd outbreaks in many dairy producing areas throughout the world [3]. In Egypt *M. bovis* was isolated from clinical, subclinical and tank milk of bovine dairy herds at different Governorates [4] and [5]. Since there is no effective antimicrobial therapy or mass screening method for detecting infected animals and / or herds, prophylactic vaccination could prove 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 was isolated 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.

**PCR procedure:** was performed on a Bio-Rad cycler (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 in PBS 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.


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 boosting after 2 weeks and challenged 2 weeks later with 0.2 ml(10^8 cfu / ml) S/C of field *M. bovis*(Egy-Bu-6-DK-12).
2) Group B (vaccinated / not challenged): 4 rabbits were vaccinated 2 weeks later S/C with 0.5 ml* M. bovis*. Vaccine then boosting 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/C after 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](image)

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

Two localities in Fayoum Governorate (farm1 represented by 56 animals, farm2 represented by 80 animals) three herds were chosen from each farm, and in Behera Governorate, one farm represented 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 PCR continued 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

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Fayoum Governorate</th>
<th>Behera</th>
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<tbody>
<tr>
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<td>Farm1</td>
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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, 5 and 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 contagious disease that results in milk loss and culling of infected animals. Several species of Mycoplasma have been associated with mastitis (M. bovis, M. californicum, M. canadense, M. bovigenitalium, M. alkalescens, M. 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 all the vaccinated animals until 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 aseptic 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 saponin 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 6 months. The vaccine proves to be safe, efficacious and immunogenic.

6. Acknowledgment

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