# Preparation of autogenous bivalent vaccine for *M. bovis* and *M. bovigenitalium* in Egypt

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**Abstract:** In view of the decreasing effectiveness of antibiotics in controlling *Mycoplasma* infections and no vaccine is available against *Mycoplasma* in Egypt, the need for reliable vaccines has become even more urgent. The present study tried to prepare two bivalent autogenous vaccines (saponised and formalized vaccines) able to protect against *M. bovis* and *M. bovigenitalium*. The prepared vaccines were experimentally injected in groups of rabbits and challenged with virulent strain of *M. bovis* and *M. bovigenitalium*. Both saponised and formalized vaccines were able to protect rabbit against *M. bovis* and *M. bovigenitalium*. Meanwhile saponised vaccine was safe and more potent than formalized vaccine. Experimental work had shown that a vaccine inactivated with saponin can protect in the face of a large *Mycoplasma* challenge and was highly immunogenic.

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**Key words:** *M. bovis*, *M. bovigenitalium*, saponin, *Mycoplasma* vaccine, formalized vaccine.

## 1. Introduction:

*Mycoplasma* species are highly contagious pathogens cause a serious problem on dairy farms. *M. bovis* is a small, cell-wall less bacterium causing a number of diseases including bronchopneumonia, meningitis, otitis media, arthritis, mastitis, abscesses, keratoconjunctivitis and a variety of other diseases in cattle worldwide (Stipkovits *et al.,* 2005; Van der Merwe *et al.,* 2010; Maunsell *et al.*, 2011). *M. bovis* and *M. bovigenitalium* have the ability to colonize the reproductive tract and produce salpingo-oophoritis and reproductive failure in cattle (McEntee, 1990). Both mycoplasmas have been isolated from semen and are transmitted by natural breeding and by artificial insemination (Bielanski *et al.,* 2000). Recently Roy *et al.* (2008) recorded a first report of an intramammary infection caused by *Mycoplasma bovigenitalium* in a 7-weeks old Holstein calf.

Treatment of *Mycoplasma* diseases is difficult since *Mycoplasma* species lack a cell wall, which differentiates them from bacteria and is thus resistant to some commonly used antibiotics. Despite the seriousness of *Mycoplasma* diseases, there are few effective vaccines to combat them today. Indeed, those that are available are whole-cell vaccines, some of which are semi virulent, provide only transient or partial immunity and often induce unpleasant side effects. Furthermore, and alarmingly, attempts at vaccine improvement have often led to exacerbation of diseases, due to their immunopathological nature (Nicholas *et al.,* 2009). Saponins are natural glycosides of steroid or triterpene which exhibited many different biological and pharmacological actions such as immunomodulatory, antitumor, antiinflammatory, molluscicidal, antiviral, antifungal, hypoglycemic, hypocholesterolemicb (Lacaille-Dubois, 2005). The aim of the present study was to establish an early control method for bovine *Mycoplasma* diseases specially respiratory and genital form using saponised and formalized vaccines.

## 2. Materials and Methods

### Identification of *Mycoplasma* isolates:

Two *M. bovis* and two *M. bovigenitalium* isolated from El-Kaliobia and Giza governorates were identified using the conventional methods, Immunoblotting (Towbin *et al.,* 1979) and polymerase chain reaction (Sambrook *et al.,* 1989) using the following Oligonucleotide primers used for detection of *M .bovis* and *M. bovigenitalium:*

### Preparation of autogenous inactivated vaccines:

### 1. Preparation of *Mycoplasma* culture:

Local *M. bovis* and *M. bovigenitalium* isolates were inoculated into Modified Hay Flick's medium (Rosendal, 1994) for 48 hrs at 37º C, 5-10% $CO_2$. The broth cultures were grown on Modified Hay Flick's agar medium to check purity of *Mycoplasma* suspension. The suspension was centrifuged at 10000 rpm/min and washed 3 times with PBS.

### 2.Preparation of formalized inactivated *Mycoplasma* vaccine:

*Mycoplasma* suspensions were inactivated by 1 % formalin (38% analytical reagent grade) at 37º C for

24-48 hrs. After completion of activation, all isolates were mixed together.

| Primer designation | Specificity | Length | Sequence (5̀-3̀) | Amplified Product size (bp) | Annealing temperature | Reference |
|---|---|---|---|---|---|---|
| MBsf-MN | *M .bovis* | 19 | CCA GCT CAC CCT TAT ACA T | 442 | 52 ºC/1 minute | (Pinnow *et al*., 2001) |
| MBsr-MN | | 19 | TGA ATC ACC ATT TAG ACC G | | | |
| MBsr-MN | *M. bovigenitalium* | 18 | ACC ATG GGA GCT GGT AAT | 928 | 56ºC/1 minute | Gene Bank # AY 780797 |
| MBmr-MN-927 | | 18 | TTC TTA CTT CTA AAG TAT | | | |

**3. Preparation of saponin inactivated *Mycoplasma* vaccine:**

*Mycoplasma* suspensions were inactivated by saponin (Sapogenin glycosides, Sigma) at 2mg/ml overnight at 37º C. After completion of activation, all isolates were mixed together.

**4. Preparation of emulsion for vaccines:**

An oil emulsion vaccine with an aqueous phase was prepared. Mineral oil (Risella 17 oil) and SPAN 80 (Biobasic) were used as adjuvant (oily phase) while Tween 80 (HIMEDIA) and physiological saline were used as aqueous phase emulsifier.

**Quality control of the prepared vaccines:**

The prepared vaccines were tested for purity, sterility, completion of inactivation and safety test according to Standard International Protocols as described by the British Veterinary Codex (1970).

**Challenge test** (Nicholas, 2002):

Only 0.2 ml of *Mycoplasma* isolates suspension contain 1.2 x $10^5$ cfu/ml were administrated by aerosol administration into vaccinated and unvaccinated rabbits at day 43 of designed experiment.

**Experiment design:**

Six groups of New Zealand rabbits (7-9 weeks old) weighting 1.5 kg were housed separately and vaccinated s/c. These groups represented as:

1- Group A (vaccinated / challenged): 3 rabbits were inoculated with formalized inactivated vaccine then boostering after 3 weeks and challenged 3 weeks later with aerosol administration of virulent mixture of *M. bovis* and *M. bovigenitalium* on consecutive days.
2- Group B (vaccinated / challenged): 3 rabbits were inoculated with saponin inactivated vaccine then boostering after 3 weeks and challenged 3 weeks later with aerosol administration of virulent mixture of *M. bovis* and *M. bovigenitalium* on consecutive days.
3- Group C (unvaccinated / challenged): 3 rabbits were challenged 3 weeks later with aerosol administration of virulent mixture of *M. bovis* and *M. bovigenitalium* on consecutive days.
4- Group D (vaccinated / not challenged): 3 rabbits were inoculated with formalized inactivated vaccine then boostering after 3 weeks and not challenged. These were monitored for adverse effects and antibody response.
5- Group E (vaccinated / not challenged): 3 rabbits were inoculated with saponin inactivated vaccine then boostering after 3 weeks and not challenged. These were monitored for adverse effects and antibody response.
6- Group F (unvaccinated / not challenged): 3 rabbits as control group.

**Estimation of humoral immune response among the vaccinated group using:**

**1. Enzyme Linked Immunosorbent Assay ELISA** (Maunsell *et al.,* 2009):

**2. Micro-agglutination test** according to Harry and Yoder (1982):

**Estimation of anemia and carcinogenic effect of the prepared vaccines:**

Blood and serum samples collected from vaccinated and unvaccinated groups were examined for detection of anemia tumor factor (CA125, CA19.5, CEA and AFP) using ELecsys 1010 (ROCHE) and IMMULITE (DPC) kits at the end of the experiment.

**3. Results and Discussion**

*Mycoplasma* species causes some of the most serious and economically most costly diseases of cattle. In Egypt *M. bovis* and *M. bovigenitalium* were isolated from bovine samples with percentage of 2.7 % and 1.7 % respectively (EL-Jakee *et al.,* 2008). Surprisingly, no vaccines are currently available in Egypt for protection against bovine mycoplasmae in the field. Therefore a critical need to develop

improved strategies for prevention of mycoplasmae associated disease. In the present investigation *M. bovis* and *M. bovigenitalium* isolates were identified according to Quinn *et al.* (2002) and confirmed to be *M. bovis* and *M. bovigenitalium* using PCR and immunoblotting as shown in Photos (1 and 2). Two autogenous bivalent vaccines (Formalized and saponin inactivated vaccines) were prepared from the collected *M. bovis* and *M. bovigenitalium* isolates. The bivalent vaccine would not only protect against the respiratory disease but might protect against other clinical manifestations, including otitis media (Friis *et al.,* 2002) and abortion (Shin *et al.,* 2003). The prepared vaccines were tested for purity, sterility and completion of inactivation according to Standard International Protocols as described by the British Veterinary Codex (1970). Also the prepared vaccines were assayed for side effects and safety by intraperitoneal administration of 1 ml of each vaccine to ten mice. None of the vaccinated mice died and the vaccines showed no reaction after vaccination.

As shown in Figure (1), there was an increase in the body weight gain in groups B (vaccinated with saponin and challenged) and groups E (vaccinated with saponin and not challenged) in comparison with other groups. No local reaction was found in all rabbit injected with saponin compared with control group. The data illustrated in Figures (2-5) revealed that rabbits vaccinated with saponised vaccine had the highest antibody titers against *Mycoplasma bovis* and *Mycoplasma bovigenitalium* compared with other groups using ELISA and Microagglutination tests. Serological result of Delafe *et al.* (2007) indicated that saponin combined vaccines can produce a specific humoral immune response to *M. agalactiae* and *Mmm* LC over 6 months with antibody levels peaking at 45 days. The results from the work of Nicholas *et al.* (2002) reported that even a single dose of vaccine prepared from saponised *M. bovis* cells may provide effective control against *Mycoplasma* induced calf pneumonia.

No local reaction or clinical sign was observed among all rabbit injected with saponin in compared with control group, also no local reaction was found in all rabbits injected with formalized vaccines except one rabbit in group D (formalized vaccine and not challenged) had slightly local reaction. After challenge, *M. bovis* and *M. bovigenitalium* were isolated from nasal cavity, tracheal bifurcation, lung, vagina and joint fluid of rabbits in group C (unvaccinated and challenged). Pneumonia and swelling of joints were seen in the same group. Quillaja saponins have serious drawbacks such as high toxicity, undesirable hemolytic effect and instability in aqueous phase, which limits their use as adjuvant in human vaccination as recorded by Marciani *et al.* (2003). Meanwhile in our experiment no anemia or carcinogenic effect could be detected among the vaccinated and unvaccinated groups using ELecsys 1010 (ROCHE) and IMMULITE (DPC) kits.

The successful use of saponin in vaccines has already been demonstrated for other *Mycoplasma* infections such as CCPP and contagious agalactia. Its effectiveness must be associated with the fact that it apparently preserves the major antigens seen in untreated whole cells (Tola *et al.,* 1999). Previously, Kensil *et al.* (1991) speculated that the high level of protection seen with the use of saponins with vaccines in mice may be caused by the ability of saponins to induce an isotype profile similar to that seen in natural immunity to bacterial infections. This work highlights the effect of using saponin vaccine on protection against *Mycoplasma* associated respiratory disease.

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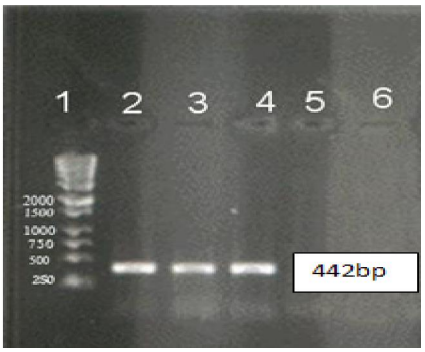
A 

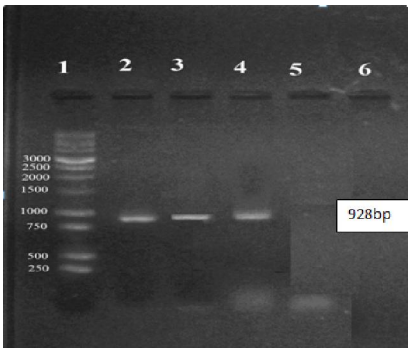

B 


Photo (1) Shows agarose gel electrophoresis showing amplification of 442 bp fragment of *M. bovis* (A) and 928 bp fragment of *M. bovigenitalium* (B).

(A)- Lane (1): DNA ladder (Sigma), Lane (2): *M. bovis* reference strain (PG45), Lane (3 & 4): *M. bovis* isolates and Lane (5): *M. bovigenitalium* reference strain (PG11). (B)- Lane (1): DNA ladder (Sigma), Lane (2): *M.*

*bovigenitalium* reference strain (PG11), Lane (3 & 4): *M. bovigenitalium* isolates and Lane (5): *M. bovis* reference strain (PG45).

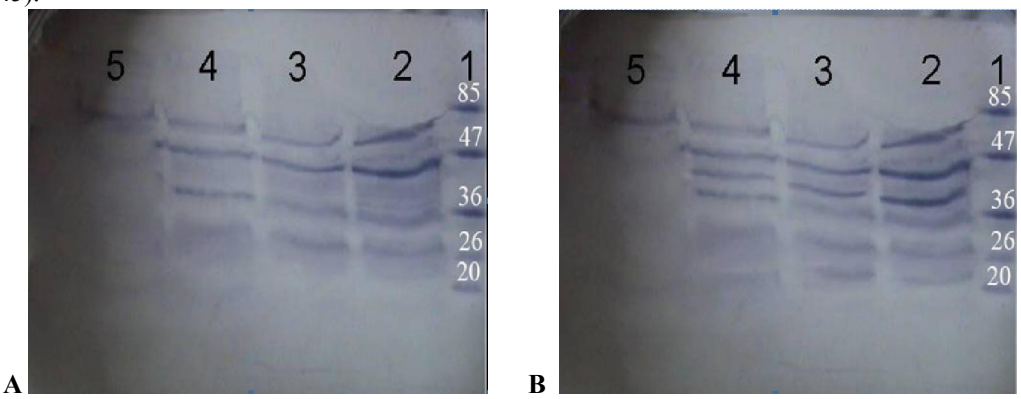



Photo (2) Shows immunoblotting against *M. bovis* (A) and *M. bovigenitalium* (B).
(A)-Lane (1): Protein marker (Ready to use) # 5M0441, Lane (2): *M. bovis* reference strain (PG45) Lane (3 & 4): *M. bovis* isolates and Lane (5): *M. bovigenitalium* reference strain (PG11). (B)- Lane (1): Protein marker (Ready to use) # 5M0441, Lane (2): *M. bovigenitalium* reference strain (PG11), Lane (3 & 4): *M. bovis* isolates and Lane (5): *M. bovis* reference strain (PG45).

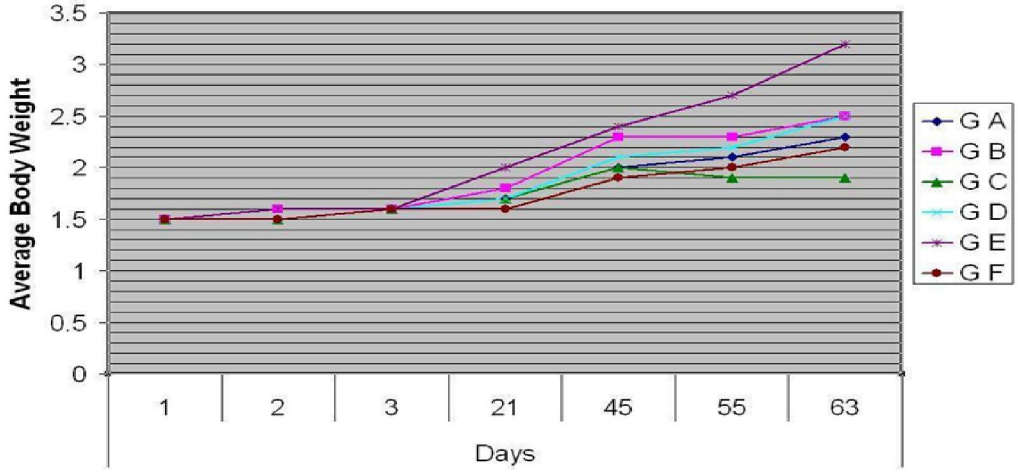



Figure (1) Body weight (kg) of vaccinated and unvaccinated rabbits.

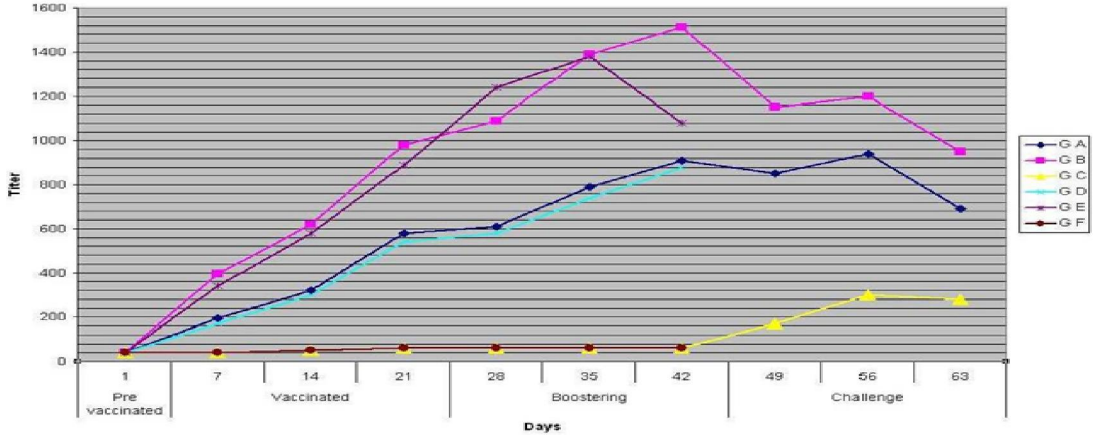



Figure (2) Antibody titers against *M. bovis* among the vaccinated and unvaccinated groups using ELISA test

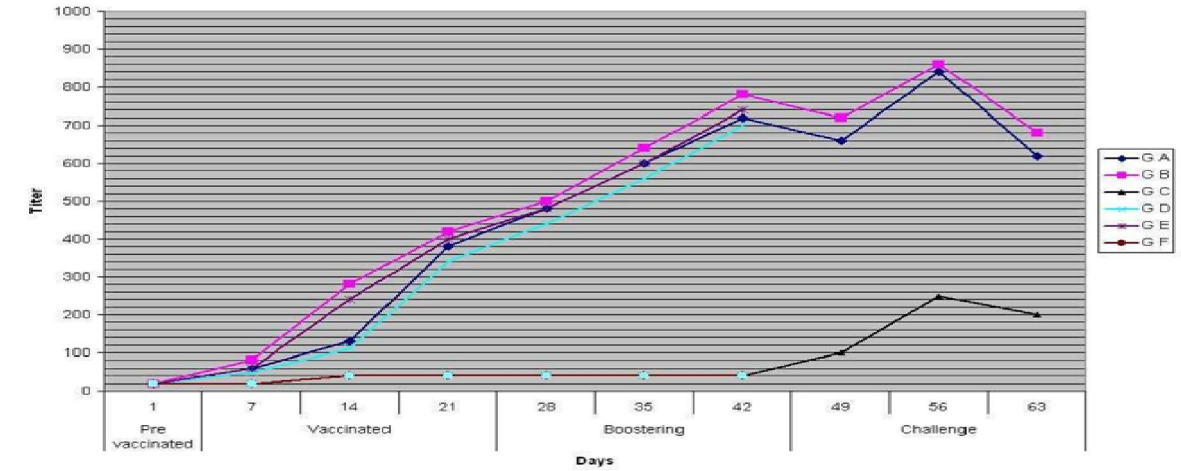



Figure (3) Antibody titers against *M. bovis* among the vaccinated and unvaccinated groups using Micro-agglutination test

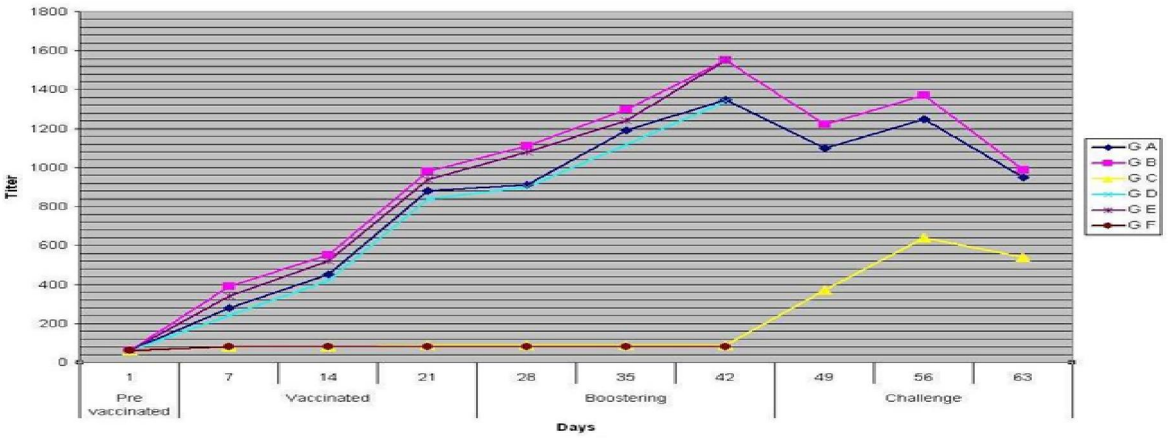



Figure (4) Antibody titers against *M. bovigenitalium* among the vaccinated and unvaccinated groups using ELISA test.

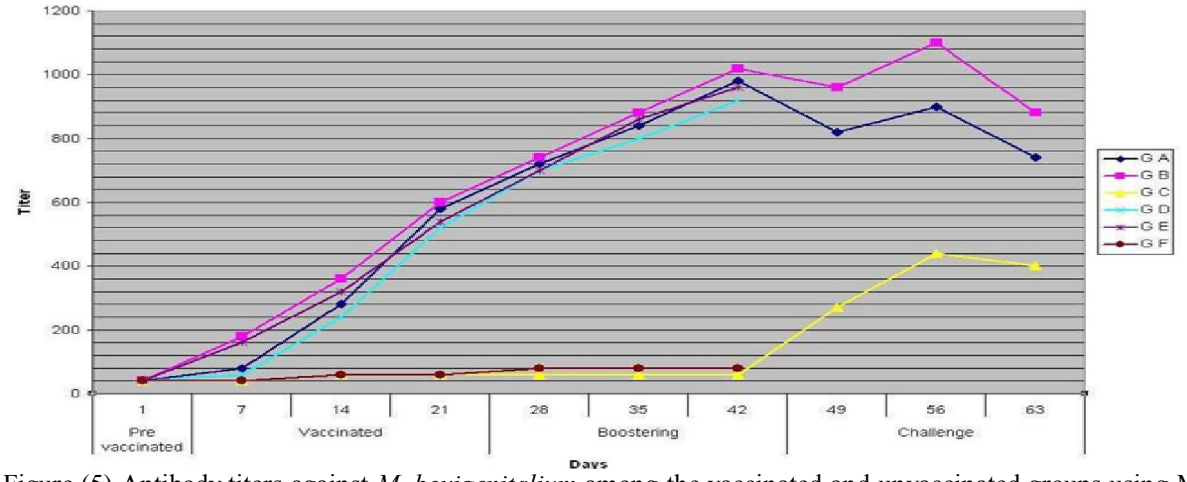



Figure (5) Antibody titers against *M. bovigenitalium* among the vaccinated and unvaccinated groups using Micro-agglutination test**.**

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