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Research Article

Protective Efficacy of the Inactivated Adjuvant Vaccines against *Mycoplasma agalactiae* Infection in Goats

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ABSTRACT

Background: *Mycoplasma agalactiae* inactivated vaccine was prepared and its efficacy in the control of contagious agalactia disease in goats was investigated. The selected vaccine candidate comprised virulent local isolates of *Mycoplasma agalactiae* that were recovered from infected goats. **Methods**: Four types of *Mycoplasma agalactiae* inactivated vaccine formulations were prepared using different inactivating agents, namely, saponin, phenol, formalin and sodium hypochlorite. With the exception of saponin-inactivated vaccines, aluminium hydroxide gel was used as the adjuvant in the other 3 vaccine formulations. The effect of these inactivators on the vaccine efficacy was determined and compared. Using a mice protection test, the saponin and phenol inactivated vaccines were the most effective, producing significant ($P<0.05$) and persistent protective antibodies in vaccinated mice as measured with ELISA. The efficacy of different vaccine formulations was evaluated under field conditions in 20 goats that were serologically negative for *Mycoplasma agalactiae* infection. Each goat was injected with two doses of the tested vaccines at four-week intervals. **Results and Conclusions:** Saponin and phenol inactivated vaccines induced significant protective efficacy and antibody response ($P<0.05$) as compared with formalin and sodium hypochlorite inactivated vaccines. Sodium hypochlorite inactivated vaccine manifested the lowest protective efficacy and antibody response in vaccinated goats.

Key words: *Mycoplasma agalactiae*, Inactivated vaccine, Goats, ELISA, Protective efficacy

INTRODUCTION

Contagious agalactia (CA), a disease characterized by mastitis, followed by agalactia, polyarthritis and keratoconjunctivitis, is primarily caused by *Mycoplasma agalactiae*. Affecting 30-85% of the animals in a short period of time, the infection spreads rapidly and causes considerable loss, including a substantial reduction in milk production and animal deaths. CA is endemic in countries in Europe, Africa, and the central and west regions of Asia, and is an emerging disease in countries on the American continent and in Japan (Azevedo *et al*., 2006; OIE, 2011; Olaogun *et al*., 2017). While the use of antibiotics for a period of five to ten days reduces the main clinical symptoms of the disease, it does not eradicate the bacteria, favoring its dissemination through the commercialization of the asymptomatic animals (Azevedo *et al*., 2006; Campos *et al*., 2013; Kumar *et al*., 2014). Control of mycoplasmosis using antibiotics is not only expansive and of low value in combating the diseases, but it also permits the recovered animal to become a carrier.

Disease control alternatives such as the use of bio-therapeutics (Marinho, 2008) and vaccines have been investigated. Vaccination against mycoplasmosis in small ruminants is of great importance in prophylaxis against serious infections including contagious agalactia, pneumonia, polyarthritis and mastitis. *Mycoplasma agalactiae* is considered to be the classic etiological agent of these clinical disorders. Studies on protein and antigenic characterization on *Mycoplasma agalactiae* isolates must be conducted to select strains suitable as candidates for vaccine preparation (De la Fe *et al*., 2007).

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The highly immunogenic surface lipoproteins of *Mycoplasma agalactiae* have a molecular weight of 45-55 kDa. The P48 lipoprotein was quantitatively the major Triton X-114 phase protein cloned. The entire open reading frame encoding for the P48 lipoprotein of *Mycoplasma agalactiae* was sequenced in order to characterize the virulent factors involved in the pathogenesis of contagious agalactia and to identify membrane proteins that could be used as potential candidates for the diagnosis and control of this infection. The mature product, P48, is responsible for adhesion of these species with the host cells (Contini *et al*., 1989; Pittau *et al*., 1990; Tola *et al*., 1997; Rosati *et al.*, 1999). The recombinant P48 is a diagnostically relevant marker of *Mycoplasma agalactiae* infection as its product plays an important role in the immune response of infected animals (Rosati *et al*., 2000).

Despite the seriousness of CA and similar diseases there are few effective vaccines to combat them today (De la Fe *et al*., 2007). 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 (De la Fe *et al*., 2007). Furthermore, and alarmingly, attempts at vaccine improvement have often led to exacerbation of diseases, due to their immune-pathological nature. Against this background, and in view of the decreasing effectiveness of antibiotics in controlling mycoplasma infections, the need for reliable vaccines has become even more urgent (Nicholas *et al*., 2009).

In Southern Europe and the Middle East, vaccines against contagious agalactia caused by *Mycoplasma agalactiae* are in widespread use (De la Fe *et al*., 2007; OIE, 2004). However, no singular vaccine has found acceptance across-the-board with no standard procedure of manufacture and evaluation being applied. Live *Mycoplasma agalactiae* vaccines not being acceptable in Europe, has led to an increased focus on the use of inactivated laboratory strains (mostly formalin-killed), with an adjuvant such as aluminium hydroxide. In parts of Italy, autogenous vaccines prepared from milk, brain and mammary gland homogenates from infected sheep materials were used for many years (Agrimi *et al*., 1999). A lack of proven efficacy and a link to severe outbreaks of scrapie in sheep and goats caused by contaminated brains has resulted in discontinuance (Agrimi *et al*., 1999). In Spain, the usage of a formalinized vaccine afforded some protection against experimental infection of goats with *Mycoplasma agalactiae* (Leon Vizcaino et al., 1995). However, even three vaccinations per year for 6 years proved unsuccessful in prevention of clinical disease following the introduction of naturally infected animals (Leon Vizcaino *et al*., 1995). Also, formalinized vaccines do not eliminate the excretion of *Mycoplasma agalactiae* in the milk (Pepin *et al*., 2001). These vaccination failures may be attributed to the high antigenic variability observed in *Mycoplasma agalactiae* field strains or to the etiological variation observed in goat herds (De la Fe *et al*., 2007; OIE, 2004). Prophylactic strategies to control contagious agalactia involve mainly the use of vaccines against virulent *Mycoplasma agalactiae.* However, the protective efficacy of such vaccines has questioned especially under field conditions (Nicholas, 1995; Bergonier *et al*., 1997).

Therefore, the present work is to investigate the protective efficacy of different formulations of inactivated adjuvant vaccines prepared from local isolates of *Mycoplasma agalactiae* that were recovered from small ruminants under desert conditions.

MATERIALS AND METHODS

Vaccine preparation

Vaccine preparation was done in two major steps: first selection of isolates, then culture in Hayflick media for vaccine preparation.

Vaccine seeds: Two field isolates of *Mycoplasma agalactiae* recovered from goats suffering severe contagious agalactia were selected for vaccine preparation. These isolates were fully characterized by bacteriological and molecular methods according to Wassif, *et al*. (2012).

Vaccine preparation: The selected *Mycoplasma agalactiae* isolates were cultured in 1000 ml of modified Hayflick medium (Cat no. ME1886, HIMEDIA, Einhausen, Germany) at 37^{o}C until mid-log phase. Then *Mycoplasma agalactiae* culture was harvested by Beckman Coulter X-15R Refrigerated Benchtop Centrifuge at 20,000 x g for 30 min and washed three times with phosphate buffered saline (PBS, pH 7.2). Finally, *Mycoplasma agalactiae* culture was resuspended in PBS and the CFU/ml of each of *Mycoplasma agalactiae* isolates was determined and adjusted to contain 5×10^{10} CFU/ml of each mycoplasma isolate accordingly to previously described method. (Rodwell and whitcomb, 1983). Four vaccine formulations were prepared using different inactivating agents. The washed *Mycoplasma agalactiae* cultures were inactivated separately with saponin (Cat No. 47036), phenol (P1037), formalin (8775) and sodium hypochlorite (425044), all from Sigma, St. Louis, Missouri, United States. With exception of saponin-inactivated vaccines, aluminium hydroxide gel was used as adjuvant in the other three vaccine formulations as previously described (Tola *et al*., 1999).

Quality control of the prepared inactivated *Mycoplasma agalactiae* vaccine formulations

Sterility test: The four prepared vaccine formulations were tested for sterility by being inoculated on Hayflick agar plates, which were incubated in a humidified chamber at 37^{o}C for 7 days and then examined with a stereomicroscope (SE305 model from AmScope, California, United States) for confirmation of sterility.

Safety test: The possible potential side effects of the prepared vaccines were assessed by the intraperitoneal administration of 1 ml of each vaccine in 10 mice and by subcutaneous inoculation of 10 ml of the vaccines in two goats for confirmation of its safety according to (De la Fe *et al*., 2007).

Evaluation of the efficacy of the prepared vaccine formulations in mice

The vaccine efficacy was evaluated by protection test in mice according to Smith (1967) by ELISA method (handmade kit) and the ELISA antibody titers optical density were measured by ELISA reader with 450 wave length according to Cassell and Brown (1983). This experiment was done in the animal house of the Faculty of Medicine (El Kasr El Einy), Cairo University.

Fifty mice were divided into five groups (10 mice in each group; each group was vaccinated with one of the 4 different inactivated vaccines by intravenous injection of 0.25 ml of each vaccine into the tail vein (saponin, phenol, formalin and sodium hypochlorite, respectively) and the fifth group was kept as a negative control. Each mouse was challenged 21 days later by intraperitoneal injection of *Mycoplasma agalactiae* strain. The test was assessed by the presence or absence of mycoplasmaemia after 24 hr of challenge. Each mouse received an intraperitoneal injection of *Mycoplasma agalactiae* field isolates in a dose of 0.1 ml (297 x 10^6) (Smith, 1967)of a 3 days culture mixed with 0.4 ml of a 5% suspension of autoclaved mucin, Sigma, St. Louis, Missouri, United States . Evaluation of vaccines by detection of mycoplasmaemia in mice (Smith, 1967) a drop of blood from each mouse was cultured in Modified Hayflick's medium. After incubation for 7 days, subculture was made on Modified Hayflick's agar medium for detection of presence or absence of mycoplasma colonies by stereomicroscope.

Evaluation of the efficacy of the prepared vaccine formulations goats

The experiment was done at the Maryout station which is belonging to Desert Research Center, Egyptian Ministry of agriculture. As all experiments in this station under the governmental licenses (agreement without challenge in goat) and ethical approval.

All goats were clinically examined by AAS before beginning of the immunization program in order to exclude past or ongoing pathologies, especially those involving the mammary glands, and to exclude any previous contact with *Mycoplasma agalactiae* infection. Twenty female goats of 6-12 month old (20-25 kg) in semi open housing with good husbandry were divided into five groups (four goats in each group). Each group was vaccinated with one of the four different inactivated vaccines (saponin, phenol, formalin and sodium hypochlorite, respectively) and the fifth group was kept as a negative control. Before and during immunization, the goats were maintained at Maryout Station–Desert Research Center, Alexandria. Two doses, each of 2 ml from the tested vaccine formulations were, subcutaneously administered with a four-week interval. Monthly blood samples were collected from all animals up to 24 weeks post-immunization. Measurement of humeral immune response using ELISA was done according to the Cassell and Brown protocol as described (Cassell and Brown, 1983).

The antibody titers and protection efficacy of the four vaccine formulations were statistically analysed using Model GLM of SAS software version 9.1.3 (SAS 2003). The statistical analysis of variance (ANOVA) test used throughout this work was according to Snedecor and Cochran (1989). The values of F test were checked under the special table of ANOVA using Degree of Freedom (D.F.) of error and source.

RESULTS

Before vaccination, none of the animals showed antibody titers against live antigen. If there were low titers in sera of these animals it may have been due to their exposure before this experiment. During vaccination a progressive increase in antibody production against mycoplasma immunogenic proteins was determined with one peak in the first couple of months. The results of immune response are given in Tables 1 and 2 and Figure 1 at different time intervals from baseline (day zero) up to week 24 at intervals of four weeks.

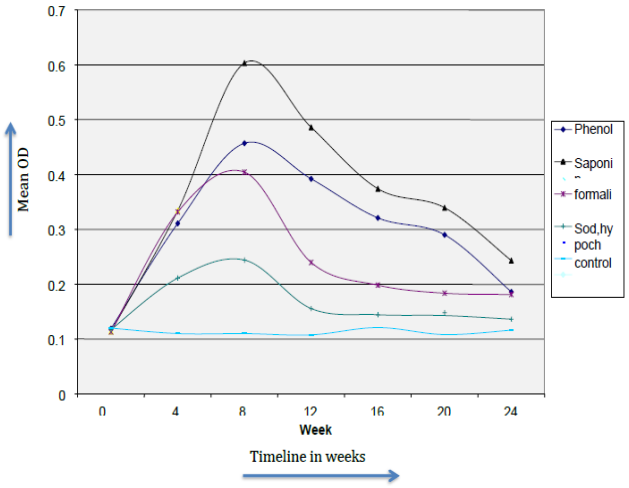

Fig. 1: Mean optical density (OD) of *Mycoplasma agalactiae* antibodies of goats vaccinated with different inactivated vaccines till week 24. Statistical analysis illustrated that there was significant (P<0.01) difference among the mean OD values of antibody level in sera of vaccinated groups as compared with control.

Table 1: Incidence of mycoplasmaemia post challenge with virulent *Mycoplasma agalactiae* strain in mice

Challenged mice	Type of inactivated vaccine				
	Group-I	Group-II	Group-III	Group-IV	Group-V
	Saponin	Phenol	Formalin	Sodium hypochlorite	Control
Survived mice (number)	10	10	10	10	10
mycoplasmaemia post challenge (24hrs)	0/10	0/10	5/10	8/10	10/10
Protection (%)	100	100	50	20	0

Incidence rate of mycoplasmaemia at 24 hrs post challenge with virulent *Mycoplasma agalactiae* strain in mice has been shown in the table. Each group (10 mice/group) has been infected with different type of inactivated vaccine.

Table 2: *Mycoplasma agalactiae* specific antibody response in sera of experimental goats

Type of inactivated vaccine	Animal group	Mean OD						
		BL	W 4	W8	W12	W16	W20	W24
Saponin	I	0.114	0.334	0.603	0.486	0.374	0.340	0.243
Phenol	II	0.119	0.311	0.457	0.392	0.321	0.290	0.186
Formalin	III	0.113	0.332	0.404	0.240	0.198	0.184	0.182
Sodium hypochlorite	IV	0.117	0.211	0.244	0.156	0.148	0.145	0.136
Control	V	0.115	0.110	0.110	0.108	0.121	0.108	0.116

Mycoplasma agalactiae specific antibody response was measured by ELISA for IgG in sera of experimental goats. The Mean optical density at measured at different time intervals; Baseline (BL), Week 4 (W4), Week 8 (W8), Week 12 (W12), Week 20 (W20), Week 24 (W24) respectively. Different animal groups (4 goats per group) are treated with inactivated vaccine in different compounds like saponin, phenol, formalin and sodium hypochlorite. The fifth is control group without any vaccine or compound.

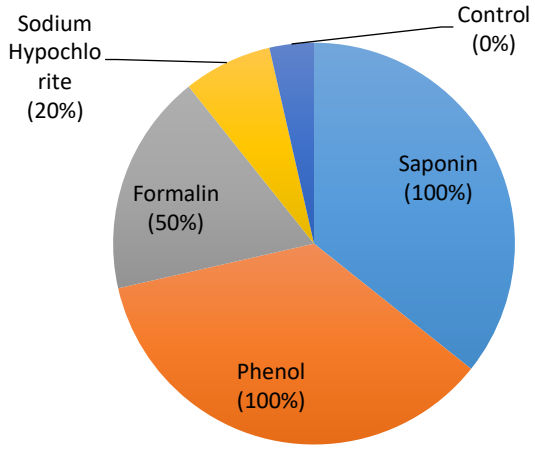

Fig. 2: Types of inactivated vaccine & protection (%)

DISCUSSION

The mouse protection test showed that *Mycoplasma agalactiae* vaccine inactivated with saponin (with saponin adjuvant) and that inactivated with phenol (with aluminium hydroxide adjuvant) gave significant protection (100% protection) after challenge with virulent *Mycoplasma agalactiae* strains. Negative mycoplasmaemia was recorded 24 hours after the challenge as shown in Table 1. On the other hand, our protection results given by *Mycoplasma agalactiae* vaccines inactivated with formalin or sodium hypochlorite and adjuvanted with aluminium hydroxide were less protective, producing a 50% and 20% protection rate, respectively. This is also correlated with other studies in mice. The immunization of mice with formalin inactivated *Mycoplasma agalactiae* elicited a relatively weak IgG response, while mixing with saponin resulted in twofold increase of total IgG level (Avramidis *et al*., 2002). These results correlated with the higher protection effect of saponin in the present work.

Other reports have found that after challenge with *Mycoplasma agalactiae*, there was no morbidity in sheep vaccinated with saponin- and phenol-inactivated vaccines but variable morbidity was reported in those immunized with formalin, sodium hypochlorite and heat inactivated vaccines (Leori *et al*., 1999; Tola *et al*., 1999). The immunoblot of saponin and phenol inactivated mycoplasma vaccines showed high integrity of P48, which are membrane proteins responsible for immunity induction in animals (Tola *et al*., 1999).

To investigate the efficacy of different vaccine formulations under field conditions, 20 goats serologically negative against *Mycoplasma agalactiae* infection were classified into five groups with four animals in each. The first four groups were vaccinated with saponin, phenol, formalin and sodium hypochlorite, respectively (two doses with four weekss interval) and the last group was kept as control. Blood samples were collected at zero time and every 4 weeks till 24 weeks from the beginning of the experiment. To measure the humoral immune response developed against *M. agalactiae*, indirect ELISA was applied. The *M. agalactiae*-specific antibody titers in vaccinated goats after 4 weeks from the first dose showed that the mean optical density (OD) values of antibody level in sera of goats vaccinated with saponin (group I), phenol (group II), formalin (group III) and sodium hypochlorite (group IV) were higher than the cut off value (Table 2 and Figure 1). Statistical analysis illustrated that there was a highly significant ($P<0.01$) difference among the mean OD values of antibody level in sera of vaccinated groups as compared with control. The result indicated the ability of inactivated vaccine used in this experiment to stimulate the humoral immune response. As shown in Table 2, the mean optical density (OD) values of antibody levels in sera of vaccinated goats four weeks after the second dose of vaccination were highly significant ($P<0.01$) as compared with control, as IgG levels peaked in the second month. Concerning the type of inactivated vaccine, there was no significant difference between saponin (I), phenol (II), and formalin (III) vaccinated groups. However, the difference between group IV (receiving sodium hypochlorite-inactivated vaccine) and the three other groups was significant, which indicates that sodium hypochlorite-inactivated vaccine has the lowest effect of stimulation of the humoral immune response. These results coincide with those reported by (Tola *et al*., 1999) who reported that inactivation with sodium hypochlorite alters the microbial immunoproteins. The ELISA results from between 12 and 24 weeks clearly showed that saponin- and phenol-inactivated vaccines had a significant ($P<0.05$) stimulation effect on the humoral immune response as compared with other vaccine formulations. The superiority of saponin and phenol as inactivating agents was recorded by many authors (Leori *et al*., 1999; Tola *et al*., 1999; De la Fe *et al*., 2007; OIE, 2008). The obtained results confirm the efficacy of saponin as an inactivating agent and may be attributable to its effect of improving the immunogenicity of vaccines (De la Fe *et al*., 2007), as its adjuvant effect combines with inactivation without damaging the immunogenic proteins of *M. agalactiae*. In addition, this eliminates the problems of mixing the inoculation dose and toxicity caused by using separate adjuvants (Tola *et al*., 1999).

Effectively, this agent has been satisfactorily used in vaccines protecting against other diseases caused by mycoplasma (Rurangirwa *et al*., 1987). De la Fe *et al*. 2007 also reported that better serological results were obtained with the phenol-inactivated vaccine which could be explained by the minimal damage produced by these agents to the immunogenic proteins of *M. agalactiae*. The drop of antibody levels (as mean OD) of formalin-inactivated vaccine after 3 months from vaccination has been discussed which correlated with the current study (Leon-Vizcaino *et al.* 1995).

Conclusions

In conclusion, in the present investigation *Mycoplasma agalactiae* vaccines inactivated and adjuvanted with saponin or inactivated with phenol and adjuvanted with aluminium hydroxide manifested significant protective efficacy. These vaccines can be of significant value in control of this infection in goats and other ruminants.

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