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Full Length Article

Evaluation of a Saponin Adjuvanted Inactivated *Mycoplasma bovis* (A Field Isolate from Cattle Lungs in Balochistan, Pakistan) Vaccine

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Abstract

An inactivated saponin adjuvanted vaccine was prepared from *Mycoplasma bovis* local field isolate and its efficacy was evaluated. Nine calves (n=9) were split in three groups (three calves in each group). Calves in group A were vaccinated with *M. bovis* saponin adjuvanted (inactivated) vaccine and challenged with live *M. bovis* strain by nasal spray at day 21 post vaccination. Calves in group B were only challenged with live *M. bovis* culture through same route and Group C was kept as control. All groups of calves were monitored for 7 weeks. The antibody profile of all vaccinated and challenged animal were assessed through IHA test. The saponated *M. bovis* (inactivated) vaccines with protein concentration (2 mg/mL) was found very effective. Any pathological lesion, mortality and any other clinical manifestation was not observed in vaccinated group of calves. Over all the immune status with a GMT (40.3) was found satisfactory with this vaccine, which was achieved 3 weeks post-vaccination and this titer has risen to a GMT (80.6) after four week and was maintained to a GMT of (64.0) on 49th days at the end of experiment.

Keywords: *M. bovis*; Vaccine; Saponin; Balochistan; Cattle

Introduction

Mycoplasmas are wall-less prokaryotes belonging to class *Mollicutes* (Ayling *et al.*, 2000). They can colonize the respiratory tract and other sites of bovine (Chazel *et al.*, 2010), caprine (Awan *et al.*, 2009), poultry (Islam *et al.*, 2011) and other animals causing many disorders.

Mycoplasma bovis is considered responsible for quarter to third of the respiratory disease in cattle (Nicholas *et al.*, 2002), arthritis, genital disorders and mastitis in large animals (Caswell and Archambault, 2007; Abubakar *et al.*, 2012). Ineffectiveness of antibiotic therapy to control *M. bovis* has diverted some attention towards vaccination. Currently very few vaccines against *Mycoplasmas* in cattle are available in the world (Howard *et al.*, 1987; Bowland and Shewen, 2000; Urbaneck *et al*., 2000; Nicholas *et al*., 2002).

Different adjuvants are commonly being used in development of vaccines (Glenny *et al.* 1926; Freund *et al.*, 1937). Saponin an extract from the bark of the South American tree *Guillaia saponaria* has been used both as inactivant and adjuvant (Kensil *et al.*, 1991) and is recommended for use in food animals (Mulira *et al*., 1988).

Numbers of serological test are being used for the detection of *M. bovis* antibodies from serum and other body sites (Brank *et al*., 1999). The indirect hemagglutination (IHA) test has been used successfully by many workers for the detection of antibodies against *Mycoplasma* species (Gagea *et al*., 2006).

There are approximately 2.25 million heads of cattle in Balochistan (Anonymous, 2006). Unfortunately no work regarding *Mycoplasma* involved in the respiratory disease of cattle has so for been done in this Province and no vaccine has been used previously. Recently, an extensive microbiological and molecular study was carried out on caprine pneumonia and the recovered *Mycoplasmas* were identified on the bases of biochemical, and molecular based (PCR, PCR-RFLP) tests (Awan *et al*., 2009; 2010).

Present study was therefore designed to produce and evaluate a vaccine against *M. bovis* isolated from cattle lungs. Post vaccinated and post challenged antibody response was detected through IHA test. Clinico-pathological and bacteriological finding in challenged animal were also monitored.

Materials and Methods

Preparation of *M. bovis* Killed Vaccine

The culture of *M. bovis* (stored at -80^{o}C) isolated from cattle lungs, identified through PCR was reactivated in Modified

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Hayflick's broth at 37°C for 24 h. After activation the culture was transferred in another flask containing 100 mL of modified Hayflick's broth and incubated at same temperature and time. Later this activated culture was used for inoculation of one liter modified Hayflick's media @ 10% for mass cultivation. An agar plate was also inoculated to observe the growth and colony morphology. Both the flask and plate were incubated at 37^0 for 48 h in 5% CO_2 environment. After 72 h the growth of Mycoplasma was counted through the standard plate count method as used by (Miles *et al*., 1938) and used for vaccine production.

Inactivation of *M. bovis*

Mycoplasma activated stock culture was centrifuged at 10,000 g for 30 min, washed once in normal saline (pH 7.2) and was re-suspended in sterile normal $1/50^{th}$ of original volume. Filter sterilized saponin (sigma) was added @ 2 mg/mL to cell suspension and incubated for 1 h at 37°C.

Sterility, Innocuity and Safety Test of Inactivated Stock Culture

Sterility, innocuity and safety test of inactivated stock culture of *M. bovis* cell suspension was performed as mentioned in OIE (2008) manual.

Measurement of Protein Concentration and Vaccine Dispensing

Protein concentration of saponin inactivated stock culture of *M. bovis* was measured by protein quantification kit (BCA, Cat 20831001-1, BioWorld-USA) with spectrophotometer (UV-1700 Pharmaspec-Schimadzu, Japan). Optical density (OD) of test samples and the BSA standard was measured at wavelength of 562 nm. A standard curve was made by comparing the results of protein concentration of *M. bovis* stock culture with known concentration of BSA. Protein concentration of test sample was obtained from this curve. Saponin (Merck-Germany) was used as adjuvant @ 2 mg/mL protein concentration of *M. bovis* cell.

Experimental Design for Vaccine Trial

A nine (n=9) apparently healthy male calves four week old obtained from a commercial dairy farm with no previous history of Mycoplasma pneumonia and *Mycoplasma* vaccination. Calves were divided in three groups (3 calves in each). Group A was used for vaccination and challenge, Group B was used for challenged with live *M. bovis* culture by intra-nasal spray and Group C was kept as control (Table 2). All calves were housed separately, provided with premix, Lucerne hay and milk replacer daily. All calves were kept for four weeks post inoculation after which they were examined.

Vaccination and Experimental Challenge with Virulent *M. bovis* Field Strain (locally isolated)

Calves in group A were vaccinated with 1 mL of saponin adjuvanted vaccine (containing 2 mg/mL protein concentration) subcutaneously and all the calves in Group A and B were challenged with 10 mL of live *M. bovis* culture at day 21 by intranasal spray. Calves in group C were kept as un-inoculated control and were given only 10 mL of normal saline through nasal spray. All the calves were observed for any local reaction at the site of inoculation or death.

Clinical Examination and Collection of Blood Sample

Challenged and vaccinated calves were monitored for period of 28 days post-challenge. Clinical assessment was performed daily and a clinical scoring system was developed in which calves were scored in six categories detail is given below:

General appearance: 0= normal behavior, 1=slightly depressed (get up when stimulated), 2= depressed and unable to rise with out external stimulus.

Nasal discharge: 0=absent, 1: mild, 2=excessive.

Cough: 0= absent, 2=mild, 3=severe.

Arthritis: 0=absent, 1=slightly swollen, 2=swollen and painful, 3=very painful and unable to stand.

Feed intake: 0= normal, 2=slightly less intake, 3=total off feed.

Rectal temperature: 0= < 103□F, 1=103 to 104.9□F, 2:=>104□F.

Gross lung lesion at necropsy: 0= no lesion, 1= small area of lung effected in one lobe, 2=both lung effected, 3= more than half of lung effected and water in thoracic cavity.

Microbiological Examination

All the challenged and vaccinated calves were sacrificed after 28 days (four weeks) at the end of experiment and postmortem examination was performed. Samples were obtained from different organs and recovered *Mycoplasmas* were identified through PCR.

Measurement of Antibodies by IHA Test

Blood samples (5 mL) were collected for antibody detection by IHA after every week from calves in all group serum was separated and antibodies were quantified by indirect haem-agglutination (IHA) test as described by Cho *et al.* (1976).

Data Analysis

Microsoft Excel, 2003 were used to calculate GMT value.

Results

Viable Count and Protein Concentration and Inactivation of Stock Culture

Over all a viable count of 10^8 CFU/mL from stock culture

was obtained and protein concentration was 3 mg/mL. Washed cell of *M. bovis* were successfully inactivated with 2 mg/mL filter sterilized saponin at 37°C for 1 h.

Sterility, Innocuity and Safety Tests

Inactivated culture of *M. bovis* was found sterile, completely inactive and no adverse effect of vaccine was noticed in the guinea pigs inoculated with saponin adjuvanted killed *Mycoplasma bovis* stock culture.

Clinical Assessment

All (n=9) calves were found negative for *Mycoplasmas* through PCR and Serum samples from all calves were found seronegative against *Mycoplasm bovis* through indirect haem-agglutination test (IHA) pre vaccination.

All the calves in Group A, B and C after trial of vaccine were observed for the appearance of clinical signs and no abnormal clinical sign were observed in any animal before challenge up to day 21.

After challenge with *M. bovis* clinical signs like cough and nasal discharge, increase temperature, lungs lesions and arthritis were recorded in Group B. In this group respiratory signs were not so sever but sever arthritis was observed in all calves. In addition lameness, local pain and accumulation of fluid in joints (Fig. 1) were also observed.

All the calves in group A were found generally healthy, only calf no A1 and A2 were slightly depressed and calf A1 showed slight cough post challenge. Calves in group C did not show any sign. Total clinical assessment was high in Group B with a total score of 31 as compared to Group A with total score of 5 points (Table 2). No considerable rise in rectal temperature was observed in Group A and C. Rise in temperature was observed in group B (Table 2).

Body weights of calves before the start of the experiment ranged from 28 kg to 33 kg. No significant difference in Average body weight gain of calves in all groups was observed before and after two weeks of challenge. Decrease in weight gain was observed in group B as compared to other two groups (Graph 1). After one week of challenge one calf in Group B became totally off feed and died at 29 day. Other two calves in this group developed a pain full arthritis and feed intake was also found decreased and feed refusal progressed with the time and became worse at the end of experiment.

Post Mortem Finding

After challenge *M. bovis* was detected from nasal cavity of all calves (in group A) and trachea of one calf in Group A. while it was detected from nasal swab, trachea, lungs and joints of all calves and also from some of other organs in Group B. (Table 3). *M. bovis* isolated from different body sites of experimental animals was confirmed through PCR (Fig. 2). Gross lung lesions were not so severe except

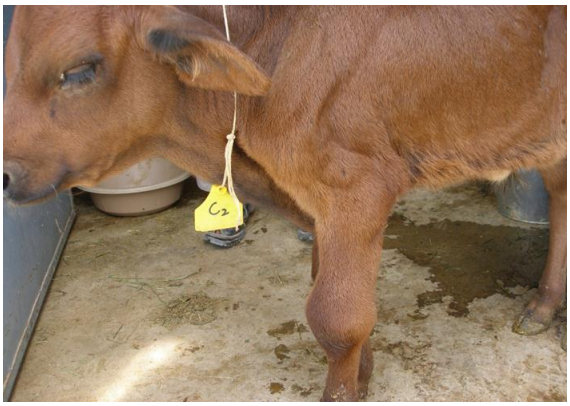


Fig. 1: Arthritis observed in calf after challenge (Swollen knee) of the calf)

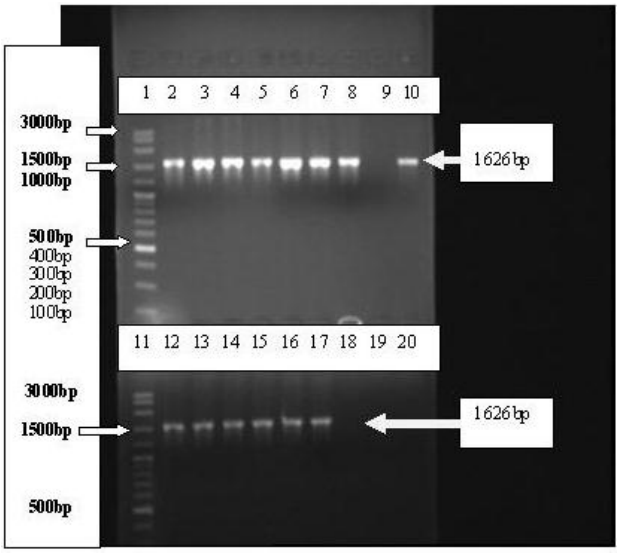


Fig. 2: Gel electrophersis of the PCR product showing amplified product of 1626bp of *M. bovis* Lane 1 and 11 molecular markers (3000bp DNA ladder) (vivantis), Lane 10 positive control (*M. bovis local isolate*) Lane 2 to 8 and 12 to 17 field strains of *M. bovis*, Lane 9 negative control

unilateral pneumonia and some area of red consolidation was observed in lungs in Group B.

Quantification of Antibodies through IHA Test

Serum samples from Group A, Group B and Group C were collected on 7, 14, 21, 28, 35, 42 and 49 days post vaccination and subjected to IHA for antibody titer.

All the serum samples from the calves in group A achieved an antibody titer with a geometric mean titer (GMT) 40.3 after 3rd week post vaccination. These calves were challenged after three weeks at day 21. The antibody titer increased to a GMT of 80.6 after one week and was maintained to a GMT of 64.0 after 4 weeks post challenge at the end of experiment. While in group B, which was

Table 1: Clinical score observed in different groups

Group	Calf N0	General appearance	Cough	Nasal discharge	Arthritis	Temperature	Gross lung lesion
A(Vaccinated and challenged)	1	1	1	0	0	1	0
	2	1	0	0	0	0	0
	3	0	0	0	0	1	0
	Total	2	1	0	0	2	0
B (Challenged only)	1	3	1	1	2	1	1
	2	2	1	0	2	1	1
	3	4	2	1	3	2	3
	Total	9	4	2	7	4	5
C (Control)	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	Total	0	0	0	0	0	0

Table 2: Temperature recorded in calves during experiment and Mycoplasma recovered from different body sites of experimental calves

Group	Animal no (route of inoculation for challenge)	Maximum temperature recorded°F	Days (D/S)	*Mycoplasma* recovered site at postmortem						
				N	T	L	S*	L	K	J
A	1 (NS)	103.4	49 (S)	+	-	-	-	-	-	-
(Vaccinated and challenged)	2 (NS)	103.0	49 (S)	+	+	-	-	-	-	-
	3 (NS)	103.6	49 (S)	+	-	-	-	-	-	-
B (Challenged only)	1 (NS)	104.4	49 (S)	+	+	+	+	-	-	+
	2 (NS)	104.2	49 (S)	+	+	+	-	-	-	+
	3 (NS)	104.6	29 (D)	+	+	+	+	-	-	+
C (Control)	1 (NS)	103.0	49 (S)	-	-	-	-	-	-	-
	2 (NS)	102.6	49 (S)	-	-	-	-	-	-	-
	3 (NS)	102.4	49 (S)	-	-	-	-	-	-	-

NS: Nasal spray N: Nose T: Trachea L: Liver K: Kidney
J: Joints D: Died S*: Spleen S: Spleen

Table 3: Geomean (GMT) antibody titres recorded in *M.bovis* vaccinated and challenged calves by Indirect Haem-agglutination Assay (IHA)

Group	Sample No. (calves)	IHA antibody titers at post inoculation week						
		1	2	3	4	5	6	7
A (Vaccinated and challenged)	1	1:8	1:32	1:64 challenged	1:128	1:128	1:128	1:128
	2	1:4	1:16	1:32	1:64	1:64	1:32	1:32
	3	1:8	1:32	1:32	1:64	1:64	1:64	1:64
	GMT	6.3	25.4	40.3	80.6	80.6	64.0	64.0
B (Un-vaccinated and challenged)	1	1:2	1:2	1:2	1:16	1:64	1:64	1:32
	2	1:2	1:2	1:4	1:32	1:32	1:64	1:32
	3	1:2	1:2	1:4	1:16	D	D	D
	GMT	2.0	2.0	3.2	20.2	45.3	64.0	32.0
C (Placebo)	1	1:2	1:2	1:2	1:2	1:2	1:2	1:2
	2	1:4	1:4	1:4	1:4	1:4	1:4	1:4
	3	1:2	1:2	1:2	1:2	1:2	1:2	1:2
	GMT	2.5	2.5	2.5	2.5	2.5	2.5	2.5

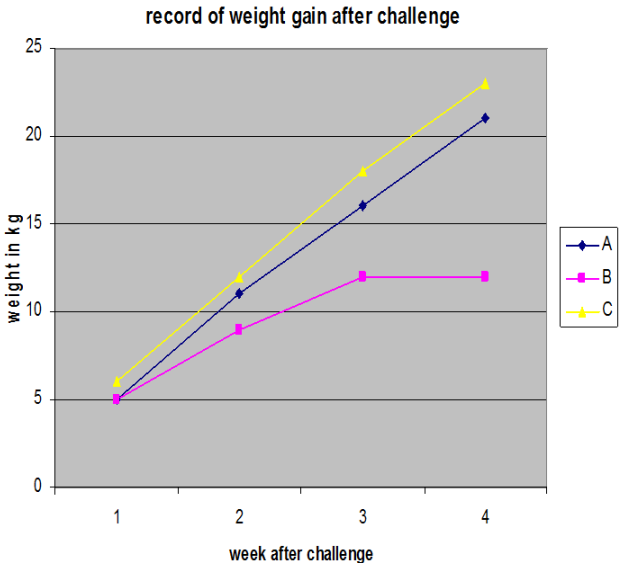
D: Died GMT: geometric mean titer

only challenged at day 21 with *M. bovis* and no vaccination was done, the antibody titer with a GMT of 20.2 was recorded after fourth week and maximum titer was recorded up to a GMT of 1:64 after sixth week and dropped to a GMT of 1:32 at the end of experiment (cut-off value of ≥1:16 was considered positive for *M. bovis*) (Table 3).

Discussion

Mycoplasma bovis is the second most pathogenic *Mycoplasma* species in the respiratory tract of cattle after *Mmm* SC (Nicholas *et al*., 2000). It causes major economic losses by producing pneumonia (Pfutzner and Sachse, 1996). Chemotherapy does not work effectively in *Mycoplasma* diseases therefore a focus on vaccination must be done to control *Mycoplasma* infection (Howard *et al*., 1987). In present study a saponin adjuvanted (inactivated) *M. bovis* (locally isolated field strain) bactrim was evaluated in calves. Saponin has been reported as a successful adjuvant by many researchers (Kensil *et al*., 1991; Nicholas *et al*., 2002).

A total of 9 calves divided in three groups (Table 2) were used for evaluation of vaccine. Calves in group A did not show any adverse clinical sign as compared to group B. After challenge at day 21 with *M. bovis* clinical sign such as



Graph 1: Weight record in all groups

cough, nasal discharge, increase temperature, lungs lesions, severe arthritis and decrease weight gain were recorded in Group B (challenged only). These type of clinical signs were also reported by others (Stipkovits *et al*., 2001; Lamm *et al*., 2004). In group B although respiratory sign were not so sever but severe arthritis was observed in all calves with lameness, local pain and accumulation of fluid in joints with swelling which is in agreement with (Stipkovits *et al*., 1993; Butler *et al*., 2000). Lower gain in body weight was observed in group B as compared to other two groups two week after challenge. This is in agreement with (Van Donkersgoed *et al*., 1993; Tschopp *et al.*, 2001) who also reported that *M. bovis* infection results in decrease weight gain.

At postmortem *M. bovis* was detected from nasal cavity of calves and from trachea of one calf in Group A and from different internal organs (trachea, lungs, spleen, joints and nasal cavity) of the calves in group B. which is in agreement with the findings of (Poumarat *et al.*, 1996; Romvary *et al*., 1977) who reported haemtogenic spread of *M.bovis*. Gross lung lesions were not so severe except unilateral pneumonia and some area of red consolidation was observed in all calves in Group B. While no gross lesions were observed in group A and C.

Antibodies produced against inactivated *M.bovis* saponin adjuvanted vaccine were evaluated by indirect haemagglutination test. In group A an antibody titer with a GMT 40.3 was recorded after 3rd week of the vaccine inoculation. These calves were challenged after three weeks at day 21st. The antibody titer increased to a GMT of 80.6 after one week post challenge and was maintained to a GMT of 64.0 after 4 weeks post challenge at the end of experiment. While in group B an antibody titer with a GMT of 20.2 after third week and maximum titer was recorded up to a GMT of 64.0 after sixth week and dropped to a GMT of 32.0 at the end of experiment (cut-off value of≥1:16 was considered positive for *M. bovis*). Calves in group C showed a negligible and stable titer through out the experiment. The indirect hemagglutination (IHA) test has been used for the detection of antibodies against Mycoplasma species and was found sensitive test in detecting antibodies (Cho *et al*., 1976; Rahman *et al*., 2003).

In conclusion, *M. bovis* causes major economic losses by producing pneumonia, arthritis and mastitis in cattle world wide. In present study A saponin adjuvanted vaccine prepared from *M. bovis* local field isolate was found successful when evaluated in calves. All the calves used in trial were monitored post vaccination and challenge. The antibody profile of all vaccinated and challenged animal were assessed through IHA test. The saponated *M. bovis* (inactivated) vaccines with protein concentration (2 mg/mL) was found very effective. Any pathological lesion, mortality and any other clinical manifestation was not observed in vaccinated group of calves.

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