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Evaluation of combined adjuvant vis-à-vis single adjuvant for development of caprine pleuropneumonia vaccine in goats using indigenous *Mycoplasma mycoides* subsp. *capri* isolate

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ABSTRACT

The disease caprine pleuropneumonia is endemic and is of serious concern in goats in India. The etiological agent *Mycoplasma mycoides* subsp. *capri,* widely prevalent in Asia and other parts of the world. The organism causes pneumonia outbreaks in goats. In this experiment, an attempt was made to develop killed *Mycoplasma mycoides* subsp. *capri* vaccine. The trial was conducted with saponin (vaccine A) and saponin + aluminium hydroxide (vaccine B) as adjuvants incorporating 1.5 mg protein of sonicated *Mycoplasma mycoides* subsp. *capri* antigen in each preparation. Upon challenge at 12 month post inoculation, vaccine A and B produced 75 and 100% protection, respectively. The result indicated the possibility of vaccine B as future candidate vaccine for caprine pleuropneumonia. However, large scale field trial is warranted. Further, the vaccine was found to be superior over in use vaccines in terms of per cent protection, extended immunity, number of immunization and minimum reaction at the site of inoculation.

Key words: Aluminium hydroxide, Adjuvant, Caprine pleuropneumonia, Goat *Mycoplasma mycoides* subsp. *Capri*, Saponin, Vaccine

Mycoplasma mycoides subsp.*capri (Mmc)* is the etiological agent of caprine pleuropneumonia in the Indian sub-continent. The disease is most prevalent in Asia (Nicholas 2002) and affects the goat population throughout the world with 100% morbidity and 60–100% mortality (Radostits *et al.* 2000).

In order to control the caprine pleuropneumonia (CCPP), a few experimental killed/attenuated vaccines have been tried in India so far. Among them, majority describe only single/ individual adjuvant like aluminium hydroxide gel, saponin, incomplete Fruend's adjuvant (IFA). These vaccines provide 75–80% protection with shorter lived immunity (Rana and Srivastava 1994, Rana 1996, Srivastava *et al*. 1998, Jai Sunder *et al.* 2003, Rana *et al.* 2004). Manimaran *et al.* (2006) developed a lyophilized *M. capri* vaccine against caprine pleuropneumonia using saponified whole cell and sonicated antigen. However, they could observe only 50% protection after a year of challenge. Similarly, in other countries, several CCPP vaccines have been tried using various strains of *Mycoplasma* spp. The workers used adjuvants like complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), $Al(OH)_3$ gel, saponin, marcol-52, sorbital monooleate (Rurangirwa *et al*.1991). The CCPP vaccines are in use in African countries where *M. capricolum* subsp. *capripneumoniae* is prevalent in goats.

In India, there is hardly any report on the availability of CCPP vaccine with 100% protection and an extended period of immunity, is available. The earlier reports indicated that the oil-based adjutants are not preferred as they track in localized muscles of food animals and spoil the quality of meat. Moreover, the CFA interferes in testing the animals for tuberculosis during eradication program. Saponin acts as adjuvant and also inactivates the organism without affecting its antigencity and improves the immunogenic properties of aluminium (Rurangirwa *et al*. 1987b). Aluminium hydroxide is least toxic, good adsorbant, attracts eosinophils to the site of injection and is a better primer of the immune system (Harm HogenEsch 2002).

In view of the above facts, an attempt was made to develop CCPP vaccine using combined adjuvant and *Mycoplasma mycoides* subsp. *capri*, to evaluate the probable synergistic role of the aluminium hydroxide and saponin in immunopotentiation without a booster.

MATERIALS AND METHODS

Antigen preparation: Mycoplasma mycoides subsp. *capri* was isolated from an outbreak of goat pneumonia by Rana *et*

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al. (2003). The isolate was characterized at CIRAD-EMVT, France. The organism maintained in Microbiology laboratory of Animal Health Division, CIRG, Makhdoom, was used for antigen preparation. The culture was grown in bulk in Hank's balanced salt solution (HBSS) liquid medium (pH 7.6–7.8) using standard protocol. The cells were harvested after centrifugation in a refrigerated centrifuge at 10 000 rpm for 30 min. The cells were washed thrice with ice-cold PBS (pH 7.4) and re-suspended in it. Later the antigen was sonicated for 10 times at 10µHz; 1 min jerk with an alternate gap. The protein concentration of sonicated antigen was estimated by Lowry *et al.* (1951) and the antigen was stored at –20°C until use.

Vaccine A (sonicated *M. capri* + Saponin) and vaccine B (sonicated *M. capri* + saponin-aluminium hydroxide) were prepared separately. Vaccine A was having saponin at 3mg/ dose and sonicated *Mycoplasma mycoides* subsp. *capri* (*Mmc*) antigen at 1.5mg protein/dose. In vaccine B, the concentration of *Mmc* antigen and adjuvant saponin were same per dose except aluminium hydroxide used at 3% w/v.

Sterility and safety of vaccines

The sterility of both of the vaccines was checked over HBSS agar, blood agar and Sabouraud's dextrose agar to avoid any live *Mmc* organism, aerobic, anaerobic and fungal contamination. The inoculated plates were incubated at 37°C for 24–72 h. Moreover, both the preparations were inoculated subcutaneously at 0.2 ml/ mice, in group of 5 mice each to see the safety of vaccines by observing untoward reactions.

Experimental animals and their screening: Apparently healthy male goats (26) of Barbari and Jamunapari breeds, aged 6 months were procured from Jamunapari and Barbari goat breeding farms. The kids were maintained under intensive management system. Ten days prior experiment, all the animals were given prophylactic coverage of broad spectrum antibiotic along with the anthilmentic. All the animals were pre-screened for the absence of *Mmc* antibody and antigen. The serum samples and deep nasal swabs from all the animals were collected. The swabs were processed on HBSS liquid and solid media. The serum samples were screened for the absence of *M. capri* antibodies using indirect enzyme linked immunosorbent assay (ELISA) (Rana 1996).

Experimental design: The goats were divided into 3 groups. Groups 1 and 2 were designated as vaccine groups and comprised 9 animals each. The third group was having 8 animals and was kept as control. The animals of first and second group were inoculated separately with vaccine A (saponin) and vaccine B (saponin-aluminium hydroxide) at 1 ml/dose by subcutaneous route at the base of neck. The third group was inoculated at the same site with sterile PBS at 1ml/ dose; subcutaneously. All the animals were observed for 7 days to see any untoward reaction like rise in body temperature, reaction at the site of inoculation, coughing or other body reactions after inoculation.

Collection of serum samples and application of test: The serum samples from vaccinated animals were collected on day 0 and 3 and week 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24, and months 7, 8, 9, 10, 11, 12; post inoculation. The antibody response was monitored using ELISA (Rana *et al.* 2004). In brief, the test was standardized with standard checker board method for the selection of antigen, antiserum (antibody) and conjugate-HRPO (antigoat-rabbit) dilutions using microtitration plate .

The antibody response was checked using indirect haemagglutination (IHA) test as per Krogsgarred-Jensen (1971) and modified by Srivastava *et al.* (1991). The comparison between the groups mean was done by Student "t" test (Swedecor and Cochran 1989).

Challenge study: For potency testing of vaccines, direct challenge studies were conducted in goats. Four goats from each group were challenged at 12 month post inoculation, respectively with 4 animals of control group. All the animals were inoculated with 1.0×10^{12} CFU of virulent *M. capri* culture by intra-tracheal (I/T) route. The animals were observed up to 7 days for any untoward reactions like elevated body temperature, nasal discharge, coughing, respiratory distress and death.

RESULTS AND DISCUSSION

Sterility and safety of vaccines: After incubation of vaccine preparation over media plates for 24–72 h at 37°C, no growth was observed on HBSS agar, blood agar and Sabouraud's dextrose agar media, thereby indicating the sterility of vaccine A and B from mycoplasmal, aerobic, anaerobic and fungal contamination. Out of 10 mice inoculated, none died and did not show any unwanted behaviour, indicating the safety of vaccines.

Pre-screening of experimental goats: No isolation could be made from nasal swabs collected from the experimental animals processed on HBSS solid and liquid medium. Moreover, serum samples were free from CCPP specific antibody.

Post vaccination status of animals: All 26 goats of vaccine A, vaccine B, and control group did not show any untoward clinical symptoms like rise in body temperature, impaired movement, depression up to 1 week. These results are in agreement with earlier reports (Rurangirwa *et al.*1987b, Mulira *et al.*1988, Rana 1996, Manimaran *et al.* 2006). The former 2 group used *Mccp* vaccine with saponin, $Al(OH)_3$ and IFA as adjuvants; while the later 2 group employed saponified *Mycoplasma capri* vaccine. In the present experiment, 3 animals of group A and an animal of group B showed nodule formation (1–1.5 cm diameter) at the site of inoculation. The nodules receded to normal by third to fifth day post vaccination and which is similar to earlier findings (Srivastava *et al.* 1998). The reaction at the site of inoculation is due to the saponin, as it has detergent properties (Vanselow 1987). The reaction in group B was less, which could be attributed to incorporation of aluminium hydroxide in the

vaccine preparation, which, could have been probably adsorbed certain amount of saponin. No such event was noticed in placebo group.

Indirect haemagglutination test: Blood was collected from control group till 4 week post vaccination as there were no detectable antibodies observed.

In IHA test, the animal started exhibiting titre from first week PI in both the vaccinated group. The peak reached to its peak at second PI (596±2.77) in vaccine A and third week PI (622±2.79) in vaccine B group (Table 1). The antibody titres were observed up to 12 month PI in both the vaccinated groups. Similar result was obtained by Rana *et al.* (2004) who observed long lasting antibody response using same protein concentration (1.5mg) of *M. capri* antigen along with saponin. The findings are also in correlation with Jaisunder *et al.* (2003) who observed significant rise in IHA titre from first week and peak at sixth week PI with 2.5 mg protein conc. of *M. mycoides subsp. mycoides* type LC saponified vaccine. The rise in IHA titre at sixth week PI might be due to higher protein concentration used by them.

Our results revealed that the animals vaccinated with saponin-aluminium hydroxide (vaccine B) overall exhibited significantly ($P<0.05$) higher antibody titer (Student't' test) than the animals vaccinated with saponin (vaccine A). It may be due to the combined effect of adjuvant which enhances the immune response. Aluminium hydroxide could adsorb antigen and enable its slow release in better way which might have further provided a long lasting immunity. A similar observation of higher ($P<0.05$) antibody titer was also recorded by De la Fe Christian *et al.* (2007) who used the combined adjuvant viz. $Al(OH)_3$-saponin in preparation of vaccines for *M. agalactiae* and *Mm* LC. They observed significantly higher antibody titer in dual adjuvanted vaccinated pregnant goats and kids as compared to saponin vaccinated group. In their vaccination regime they additionally used 2 boosters in goats and 3 boosters in kids. This regime they might have adopted due to high endemicity of the disease.

Challenge studies: The protection value of the vaccines A and vaccine B was assessed by direct challenge test conducted on both the group of vaccinated goats at 12 month PI.

At 12 month challenge, in vaccine A group, 1 animal showed higher rise in body temperature (104°F) which is an indication of immunity break. No other clinical picture was observed in this animal. In vaccine B group, all the animals sustain challenge as none of the animal showed rise in body temperature above 103.0°F. In control group, all the animals exhibited high-rise in temperature up to 106.8°F with other clinical symptoms like coughing, dribbling of saliva, dyspnoea, congested eyes etc.

On 12 month challenge, animals of vaccine A group exhibited 75% protection and of vaccine B group exhibited 100% protection. Srivastava *et al.* (1998) observed 60% protection in the goats immunized with sonicated *M. capri* antigen (2.5 mg protein conc.) along with saponin at 7 months

Table 1. Mean (log_{10}) IHA titers of the goats vaccinated with *Mycoplasma mycoides* ssp. *capri* vaccines

Groups	Weeks post inoculation																							
	0	0.5*	1	2	3	4	6	8	10	12	14	16	18	20	22	24	28	32	36	40	44	48
GpA	4.44±	3.33 ±	91.11±	596±	228±	45.5±	24.4 ±	46.6±	60±	50±	153±	250±	171±	113±	26.6±	32.2±	22.5 ±	17.5±	10±	50±	50±	12.5±
(Saponin)	0.86	0.86	1.95	2.77	2.35	1.65	1.38	1.66	1.77	1.69	2.18	2.39	2.33	2.05	1.42	1.50	1.35	1.24	1	1.69	1.69	1.09
GpB	6.66±	10±	528±	351±	622±	30±	67.77±	106±	23.3±	129±	218±	165±	88.9±	34.5±	26.7±	37.8 ±	7.5 ±	37.5±	10±	52.5±	30±	12.5±
(Saponin-$A1(OH)_3$	0.82	1	2.72	2.54	2.79	1.47	1.83	2.02	1.36	2.11	2.33	2.21	1.94	1.53	1.42	1.57	0.87	1.57	1	1.72	1.47	1.09
Control	4.41±	3.97±	3.92±	4.04±	3.77±	4.21±																
	0.74	0.79	0.65	0.89	0.61	0.88																

* Day 3 post inoculation.

challenge. While at 13 month challenge, they found 80% protection which may be due to higher protein concentration. A higher degree of protection observed may be due to the use of booster dose of vaccine by them. However, we did not use booster dose to enable our vaccine preparations of more field applicability as second inoculation in field condition is rather difficult, if not impossible due to migratory nature of herd.

Although, there does not seem to be any report on use of combined adjuvanted vaccine using *M.mycoides* subsp. *capri* antigen which gave 100% protection. Manimaran *et al.* (2006) used *M. capri* vaccine with different protein concentration of whole cell (1mg and 2mg) and sonicated whole cell vaccine (2 mg protein conc.) with saponin. They could observe only 34, 67, and 67% protection respectively at 6 month and 50% protection at 12 month PI in all the 3 vaccinated group of goats.

Upon correlating the potency of vaccine A and B at 12th month challenge, vaccine B exhibited superior protection of 100% than that of vaccine A which could exhibited 75% protection. Here we can say that both these vaccines seem to be better than the earlier vaccines tried by Singh and Srivastava (1993b), Rana (1996), Srivastava *et al.* (1998), as some of these workers used large volume of the dose up to 2–2.5 ml. Moreover, they have also used the booster dose at 1 month after the primary vaccination. Manimaran *et al.* (2006) used single adjuvant with 50% protection at 1 year, and Mulira *et al.* (1988) who found 100% protection with saponin and 60% protection using aluminium hydroxide gel by giving booster at fourth month PI. We have tried to overcome these shortfalls of local site reaction, booster dosing and immunity duration by combining 2 adjuvants, viz. aluminium hydroxide and saponin and single shot application, to make its improved field applicability.

It is concluded that the combined aluminium hydroxide and saponin adjuvant produces extended immunity in goats against CCPP than saponin alone adjuvant. Further, the combination also reduces local reaction with extended period of immunity for a period of 1 year without a booster. However, large-scale validation is warranted.

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