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Control of brucellosis on an infected Murrah buffalo farm with reduced dose of *Brucella abortus* S19 vaccine administered by conjunctival route in adult animals

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ABSTRACT

Brucellosis caused by *Brucella abortus* is a serious threat to dairy farming particularly when the area is endemic with no effective vaccination programme. Abortion is the main outcome of brucellosis in pregnant animals with further complication of retention of placenta, reduced milk yield, metritis, temporary or permanent infertility, cost of veterinary services, increased inter-calving period and maintenance of unproductive animal. Owing to abortions, a contaminated environment with a high antigenic load of *B. abortus* is created on the farm in which vaccination of calves alone is not sufficient to control brucellosis. Vaccination of adult animals including calves would be an effective strategy to control abortions and bring down incidence of the disease. In the present study, attempts were made to control brucellosis on a Murrah buffalo farm where brucellosis entered about 5 years ago, and since then 86 abortions had occurred resulting into colossal losses. The strategy of testing of all animals, segregation of positive population, and decontamination of farm premises coupled with vaccination with *B. abortus* S19 vaccine of adult buffaloes as well as calves (4–8 months) was adopted. Adult buffaloes were vaccinated with reduced dose of S19 vaccine by conjunctival route and boosted after 4 months while calves were vaccinated by single standard dose of S19 vaccine by subcutaneous route. Only 6.52% (6/92) adult buffaloes became serologically positive after 1 month of conjunctival vaccination, however, these animals also became negative after 2 months. The drawback of subcutaneous vaccination of adult animals that they become serologically positive with persistent antibody titres interfering in subsequent testing was circumvented by using the conjunctival route of vaccine administration. Abortions in buffaloes were not recorded after vaccination.

Key words: Brucellosis, Buffaloes, Control, Adult vaccination, Conjunctival route

Brucellosis is a contagious bacterial disease of animals and a serious public health problem world over since its recognition in Malta in late 19th century. In India, this zoonotic disease is present in all livestock systems and increased demand for dairy products accompanied with changing and intensified farming practices has raised the concern for increased spread and intensified transmission of this infection to the human population with increased risk of disease (Smits and Kadri 2005). In animals, abortion in advance stages of gestation in females is the main outcome of infection with further complication of retention of placenta. Losses due to reduced milk yield, metritis resulting to temporary or permanent infertility, cost of veterinary services, maintenance of unproductive animals and increased inter-calving period are tremendous. The nature of brucellosis is insidious. Subsequent to abortion, most animals get pregnant and give birth to a live calf which apparently looks normal but factually the calf is weak and underweight. In addition, large quantities of bacteria are excreted in the uterine discharges and placental membranes from an infected animal during normal delivery like that of abortion which contaminates the surroundings and poses a serious threat to uninfected animals in the vicinity (Radostits *et al*. 2000). This situation is common on most dairy farms. In domestic water buffaloes (*Bubalus bubalis*) brucellosis is caused by *Brucella abortus* and is of great concern, particularly in developing and low income countries where brucellosis is not a priority disease and control program is not opted effectively due to economic considerations rather emphasis is paid on diseases which emerged as an epidemic.

Control program on brucellosis of the country permits calf-hood vaccination of 4 to 8 months old calves only but does not allow test and slaughter policy of animals (Renukaradhya *et al*. 2002). This control program also excludes vaccination of adult animals which results into truncated strategy particularly on dairy farms where animals are kept in close contact which is one of the crucial risk factors in spread of brucellosis (Radostits *et al.* 2000). In endemic

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situation, without vaccination coverage of adult animals, where antigenic load of *B. abortus* is high, the calf-hood vaccination alone is of no use because in such an environment the vaccinated calves continually exposed to virulent *Brucella* organisms and eventually become infected (Radostits *et al.* 2000). There is no information available so far in the country where vaccination of calves as well as adult animals has simultaneously been done to control brucellosis on dairy farms.

In this communication control of brucellosis on an endemically infected Murrah buffalo farm located at Hisar in Haryana is described. The strategy adopted included testing and segregation of positive animals coupled to vaccination of all negative females above 4 months of age with *B. abortus* S19 (S19) vaccine. The adult animals were vaccinated with reduced dose of S19 by conjunctival route while calves, aged between 4 and 8 months, were vaccinated with standard dose of S19 by subcutaneous route.

MATERIALS AND METHODS

Animal farm: The Government Livestock Farm (GLF) at Hisar has glorious history of raising and maintaining different species and breeds of animals since its establishment in 1809. The Sector-II of GLF reared, raised and maintained Murrah breed of water buffaloes (*B. bubalis*) for several years. In recent past, excellent milk yielding Murrah buffaloes were procured from different districts of the Haryana state and maintained at the farm. The objective of the scheme was to upgrade and conserve the genetic potential of Murrah breed and to make available good quality male calves to village Panchayats in the state. Animals were maintained under good animal husbandry conditions however, vaccination of animals against brucellosis had never been practiced at the farm.

Outbreak of abortion and collection of clinical samples: In the year 2006, abortions were recorded in buffaloes of the farm in late pregnancy. In some animals stillbirths were observed. Retention of placenta was common in such animals. Materials from aborted animals/stillbirths, which included uterine discharge, placental membranes and milk and aborted fetus etc. were subjected to bacteriological investigation of incriminating agent. Blood samples from all the animals were collected in 2006 and 2010.

Isolation of Brucella abortus*:* Isolation was attempted from clinical specimens (uterine discharge, milk) according to Alton *et al.* (1988). The specimens were inoculated onto trypticase soya agar (TSA) containing antibiotics (Farrell 1974). Prior to inoculation, the milk was centrifuged at 2000g for 15 min and top layer of cream and deposit were used for isolation. The plates were incubated at 37°C in candle jar with 5% to 10% CO_2 tension for a period of 10 days. The plates were first inspected after three days of incubation for the development of growth of smooth, entire, circular, raised, honey-coloured and translucent colonies. Preliminary examination of suspected colonies with respect to growth characteristics, Gram's staining of the isolates and agglutination reaction with the anti-*Brucella* polyclonal serum was carried out as per Alton *et al.* (1988). For identification of *Brucella* isolates up to species level, mono-specific A, M and R sera obtained from the OIE Reference Laboratory, Weybridge, Surrey, UK were used in slide agglutination test (Alton *et al.*1988).

Serological assays

The OIE prescribed Rose Bengal test (RBT) and enzyme linked immunosorbent assay (ELISA) were conducted to determine serological status of animals for brucellosis (OIE 2004).

Rose Bengal test (RBT): The RBT antigen was procured from the Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh, and the test was conducted as per their instructions. The results in RBT were considered negative or positive when there was absence or presence of agglutination, respectively.

Enzyme linked immunosorbent assay (ELISA): The smooth lipopolysacchride (sLPS) antigen and positive and negative controls for ELISA were obtained from the OIE/FAO Brucellosis Research and Reference Laboratory, Animal Disease Research Institute, Nepean, Canada. The conjugate was a monoclonal antibody tagged with horseradish peroxidase enzyme (HRP), which reacts with immunoglobulin G (IgG) of bovines, sheep and goats. Briefly, each well of flat bottom microplate (Nunc # 269620) was coated with 100µl of predetermined amounts of sLPS diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) for 18 h at room temperature and microplates were stored at –20°C or used immediately. Following 3 washing of microplate with 0.01 M phosphate buffer saline containing 0.05% Tween–20 (PBS-T) to remove unbound antigen, 100µl each of diluted serum samples, appropriate dilution of conjugates and substrate solution (0.4 mg/ml ortho-phenyldiamine dihidrochloride in 0.01 M phosphate citrate buffer, pH 5.0 containing 0.015% H_2O_2) were serially added to wells of microplate. The colour development was stopped after 5 min with the addition of 100µl of 2 M sulphuric acid to each well. The optical density (OD) was measured at 492 çm. The incubation time at each step was 45 min at 37°C and 3 washing with PBS-T were done by an ELISA plate washer after every incubation step. Dilution of 1: 100 for serum samples and controls in PBS-T were used. The dilution of conjugate used in the test was determined in preliminary trials and found to be optimal at 1: 15000. Positive and negative controls were always included on each plate while performing the ELISA and O.D. values of positive control were maintained as indicated in manual of OIE (OIE 2004). The per cent positivity (*%P*) was calculated as follows:

$$\text{Per cent positivity } (\%\ P) = \frac{\text{OD value of test sample}}{\text{Average OD value of positive controls}} \times 100$$

The criterion based on *%P* of Renukaradhya *et al.* (2001) was followed for considering a sample positive or negative for brucellosis.

Control strategy

The control strategy at the buffalo farm was executed employing following steps:

Testing and segregation of positive animals: The serum samples from all animals above 4 months of age at the farm were tested by RBT and ELISA for detecting *B. abortus* antibody and, thereafter, positive population was segregated and housed at a distant place.

Decontamination of farm premises: On the farm, calving pens and sufficient area in front of them is surrounded by 6 feet high concrete wall. This enclosure is used for keeping animals in advance stages of pregnancy. The pregnant animals are normally separated from other animals about 4 weeks prior to calving. These calving pens and enclosed area was decontaminated. For this, the entire ground area including of calving pens was first covered with dry wheat *bhusha* and then allowed to burn. The ash left out after burning was removed after 1 week time. The calving pens were white washed and disinfected with phenol (1% solution). All these steps were done in June 2010 when animals normally do not calve and environmental temperature remained high. Housing shed and milking area were also cleaned and disinfected using phenol (1% solution).

Vaccination of animals: The freeze dried S19 vaccine was procured from the Indian Immunologicals Ltd., Hyderabad. This vaccine could be used within 1 year from the date of manufacture if stored at temperatures between 2°C and 8°C and can be manipulated to obtain desired number of live bacteria in a defined volume. The calf-hood method of vaccination was followed for young animals between 4 and 8 months of age. Standard dose of vaccine comprising at least 40×10^9 live organisms/2 ml, as prescribed by the manufacturer, was given by subcutaneous route in neck region of calves. Animals above 8 months of age, except pregnant animals, were vaccinated by conjunctival route by employing reduced dose of S19 (OIE 2004). The reduced conjunctival dose was adjusted to contain 5×10^9 live organisms in 0.1 ml (100µl) volume and was placed within the conjunctival sac by means of a 1 ml plastic syringe without a needle (Nicoletti *et al*. 1978). The animals were given the second conjunctival vaccination (booster dose) 4 months later. Similar schedule of vaccination was followed to vaccinate pregnant animals after their parturition.

Monitoring of vaccinated animals: Antibody response to S19 was monitored by RBT at monthly intervals for 3 months. Both groups of animal, i.e., adult animals vaccinated conjunctivally with reduced dose of S19 vaccine and calves vaccinated subcutaneously with standard dose of S19 were examined to assess antibody response.

RESULTS AND DISCUSSION

Abortion or stillbirth in buffaloes started occurring on the farm in 2006 and continually occurred up to 2010 (Table 1). During the period, 86 abortions/stillbirths were recorded in 70 buffaloes of which 58 animals aborted once, 8 animals aborted twice and 4 animals aborted 3 times. Almost all animals aborted in third term of gestation period. Isolation of *B. abortus* was achieved from milk or uterine discharge of aborted buffaloes in the year 2006 and also in 2010. Abortions at the farm were not recorded prior to 2006.

Table 1. Occurrence of abortion in animals at Murrah Buffalo Farm, Sector-II, Govt. Livestock Farm, Hisar, Haryana during 2006 to 2010

	Year					
	2006	2007	2008	2009	2010	Total
Animals aborted	12	29	25	19	1	86

A large number of animals were found exposed to *B. abortus* infection (Table 2). Exposure to infection was ascertained by detecting antibody to *B. abortus* in 11.30% (19/168) animals in 2006 and in 31.62% (80/253) animals in 2010 (Table 3). Two serological tests, i.e., RBT and ELISA were used. ELISA was found more effective in detection of infection than RBT because sensitivity of ELISA is high (Guarino *et al*. 2001). Moreover, in endemic situation when dynamic population is examined in which animals are expected to be in different stages of infection the RBT is not successful in detecting all serologically positive animals and under such circumstances the use of ELISA is suggested (Guarino *et al*. 2001). Hence, prevalence of brucellosis in animals at the farm was determined on the basis of ELISA

Table 2. Prevalence of brucellosis in animals at Murrah Buffalo Farm, Haryana in 2006 and 2010

Year	No. of animals tested for antibodies	Positive in		Isolation attempted		Isolation achieved	
		RBT	ELISA	Milk	UD	Milk	UD
2006	168	16	19	13	5	3	3
2010	253	74	80	ND	1	—	1

RBT, Rose Bengal test; ELISA, enzyme linked immunosorbent assay; UD, uterine discharge; ND, not done.

Table 3. Age-wise prevalence of *Brucella abortus* antibody in animals at Murrah Buffalo Farm, Haryana

Year	Age	Number	Positive	Per cent
2006	4 month to 1 year	18	3	16.66
	>1 year to 3 year	54	1	1.85
	>3 year	96	15	15.62
	Total	168	19	11.30
2010	4 month 1 to year	44	11	25.00
	>1 year to 3 year	73	8	10.95
	>3 year	136	61	44.85
	Total	253	80	31.62

results in the present study.

Prevalence of antibody to *B. abortus* in animals of different age groups at the farm is shown in Table 3. Only females were included in the study. It is evident that animals of various ages were infected carrying antibody to infection. In 2006, 16.66%, 1.85% and 15.62% animals were positive in groups of 4 month to 1 year, >1 year to 3 year, and >3 year, respectively. While after a period of 4 years in 2010, 25.00% animals were positive in group 4 month to 1 year, 10.95% in group >1 year to 3 year and 44.85% in >3 year group. Buffaloes aborted year after year from 2006 to 2010. All these aborted animals were serologically positive for brucellosis.

A significant increase in per cent positivity in each group of animals was observed from 2006 to 2010. Almost three fold increase in prevalence of disease from 11.30% to 31.62% was observed at the farm after 4 years (Table 3).

Execution of control program was started with screening of animals, segregation of positive population followed by cleaning, disinfection and decontamination of premises particularly of calving pens and area surrounding the pen to reduce antigenic load at the farm. Subsequently, negative female animals, except those below 4 months of age and pregnant buffaloes, were vaccinated.

Antibody response to vaccination at an interval of 1 month up to 3 months was monitored by RBT and shown in Table 4. Out of the 33 calves aged 4 to 8 months, vaccinated subcutaneously with standard dose of S19 vaccine, 30 (90.90%) were positive in RBT after 1 month of vaccination. After 3 months of vaccination the number of RBT positive calves declined marginally to 24 (72.72%). On the other hand, after 1 month of vaccination of 106 adult buffaloes by conjunctival route with the reduced dose of S19 vaccine, only 6 (5.66%) animals were positive in RBT, and after 2 months none was positive out of 72 adult buffaloes tested.

Haryana is the native, place of Murrah breed of water buffaloes and Murrah buffalo keeping has a special role in animal husbandry. Since Murrah buffalo is an excellent milch breed with fat rich milk its farming has gained momentum in the state and other areas in the country in recent past. The Government agencies are also supporting farmers and entrepreneurs entering in dairy venture, at all levels, by providing them loans etc. However, brucellosis is the biggest threat to the dairy farming/venture in India as it causes tremendous economic losses once enters in animals at the farm. The disease is prevalent in the country and was recorded in all states and Union Territories (Renukaradhya *et al.* 2002). The situation of brucellosis like presented in this study is common on dairy farms in the state and elsewhere in the country. The dairymen/entrepreneurs are compelled to bear heavy economic losses because effective control program which included vaccination of adult animals is not in place. Moreover, farmers/ dairymen/entrepreneurs are unaware of brucellosis and they came to know about the disease only after losses had occurred.

Table 4. Monitoring of antibody response after 1 month of vaccination with standard dose of S19 vaccine in calves by subcutaneous route (calf-hood vaccination) and reduced dose of S19 vaccine in adult animals by conjunctival route

Vaccination	Route of inoculation	Number of animals	RBT positive in months		
			1	2	3
Adults	Conjunctival	106	6/106	0/72	0/72
Calves	Subcutaneous	33	30/33	ND	24/30

ND, Not done.

Initial efforts in 2006 to contain and control brucellosis at the farm by segregation of positive animals were not successful as abortions continued in subsequent years. The most likely reason appeared to be the highly contaminated environment which remained source of infection to negative but unvaccinated population. In 2010, a strategy of testing and segregation of sero-positive animals, decontamination of farm premises and vaccination of negative female animals was adopted. A reduced dose of S19 was used to vaccinate adult buffaloes by conjunctival route and animals were boosted after 4 months with same dose. The conjunctival vaccination induced a weak antibody response in few animals which was vanished within 2 months of initial vaccination.

Vaccination of adult cows with standard dose of S19 vaccine is highly successful in reducing the number of infected cows in large dairy herds in which it was impossible to institute management procedures for the control of brucellosis (Radostitis *et al*. 2000). It has been widely practiced during the 1940's and 1950's with great success (Lawson 1950), however, post vaccination antibody titres persisted for quite a long period which interfered in subsequent testing. The problem of persistent antibody titres was resolved when Plommet and Plommet (1976) showed

that following vaccination with reduced dose of S19 vaccine by conjunctival route, adult cows did not induce formation of agglutinins or complement fixing antibody. But the level of protection achieved by conjunctival route was comparable to that of standard dose given by subcutaneous route (Plommet and Fensterbank 1976). Subsequently, strategy of conjunctival reduced dose S19 vaccination was adopted in USA by Nicoletti *et al.* (1978) to control brucellosis in problem cattle herds under endemic situation. Nevertheless, OIE also prescribed conjunctival vaccination of bovines with reduced dose of S19 vaccine (OIE 2004).

The present study suggested that the conjunctival vaccination of adult population with S19 vaccine overcomes the problems of post vaccination antibody titres, which is a drawback of subcutaneous inoculation of S19 vaccine, and could be used on dairy farms endemic for brucellosis. If the dairymen wish to control brucellosis on his farm in shortest possible time then segregation of positive animals coupled with vaccination of negative female animals is needed, as was done in the present study. The conjunctival vaccination could also be used on farms which are free from brucellosis so that entry of disease through carrier animals could be avoided otherwise colossal losses could occur on entry of brucellosis on such animal farms. The strategy presented in this study to control brucellosis on an endemically infected buffalo farm could serve as a model for Government or private animal farms elsewhere in the country.

Endemic situation of brucellosis particularly on a large number of dairy farms in many parts of the country is well documented (Polding 1942, Sen 1975, Chand *et al.* 1988, Chand *et al*. 2004, Chandramohan *et al.* 1992, Isloor *et al.*1998, Dhanda *et al.* 2005, Brahmabhatt *et al.* 2009) and now the disease is spreading its wings to rural areas rapidly (Chand *et al*. 1990, Mehra 2000, Renukaradhya *et al.* 2002, Smits and Kadri 2005, Mantur and Amarnath 2008). According to 2003 Livestock Census, 283.103 million bovine (97.922 million buffaloes and 185.181 million cattle) are reared in various management practices and agro-climatic conditions in the country. This large population is at risk without an effective brucellosis control program in place. The situation of brucellosis, as was seen in the present study on a buffalo dairy farm, seems to be common on dairy farms in the country, with the result the dairymen/ farmers/breeders/ private or Government organizations suffer continuous losses which have never been assessed. With these facts and circumstances, the vaccination of all female population which should include both adults and calves, could be a successful strategy to bring down incidence of disease and stop losses. Farmers who keep one/few heads of animals, particularly in villages/rural/semi-urban areas, vaccination of all female animals would be a practical approach. Initially, this strategy could be cost intensive but in long term benefit-cost ratio is very high (Roth *et al.* 2003).

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