Experimental Study on *Brucella abortus* Strain RB51 Vaccinated Water Buffalo (*Bubalus bubalis*) Challenged with Virulent *B. abortus* Strain during Pregnancy

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Abstract: The study was conducted to determine the efficacy of *Brucella abortus* strain RB51 (RB51) vaccine in preventing abortion in pregnant water buffalo (*Bubalus bubalis*) experimentally challenged using the intravenous route, with a local pathogenic strain of *B. abortus* biovar 1 (Trinidad 1). Thirty-two female water buffalo calves aged 6-10 months were randomly divided into three groups for the vaccination trial using the subcutaneous route: Group I animals received recommended dose (RD) vaccine twice 4 weeks apart, Group II was vaccinated twice 18 weeks apart and Group III (control) received saline once. At approximately 6 months of pregnancy following natural breeding, the animals were challenged by the intravenous route with 2.5 x 10^8 to 4.4 x 10^8 colony forming units of a local strain of *B. abortus*, Trinidad 1. Blood samples were collected, pre-challenge and post-challenge, for serological assay using the BPAT and the animals were monitored for clinical signs. The bacteriological study was also performed on tissues of the dams and their calves. The frequency of abortion/stillbirths/early neonatal deaths was 55.6% (5/9), 42.9% (3/7) and 40.0% (2/5) for Groups I, II and III dams respectively ($P>0.05$; χ^2). For calves from infected dams, the frequency of isolation of *B. abortus* Trinidad 1 from the abomasal and rectal swabs was 100.0%, 80.0% and 100.0% for Groups I, II and III animals respectively ($P>0.05$). It was concluded that vaccination of water buffalo with the RB51 vaccine using the recommended dose was ineffective in preventing infection, abortion, stillbirths, and neonatal deaths.

Keywords: *Brucella abortus*, RB51 vaccine, Water buffalo, Challenge, Abortion.

INTRODUCTION

Livestock is well-known reservoirs of important zoonoses which infect humans either through direct contact or by the consumption of their contaminated animal products [1]. Brucellosis is a major zoonotic disease, widely distributed in both humans and animals, especially in the developing world [1]. The disease has major global socio-economic and public health importance [2, 3]. It also has a significant impact on the international trade of animals and their products [4].

Several species of *Brucella* are capable of causing infertility, abortion and other conditions in livestock and man [5, 6]. In cattle (*Bos taurus* and *Bos indicus*) and domesticated water buffalo (*Bubalus bubalis*), brucellosis is a result of infection with *Brucella abortus* [2, 7]. *B. abortus* causes reduced fertility and abortion in cattle [7] and water buffalo [8] as well.

Vaccination has played a significant role in the control and subsequent eradication of bovine brucellosis in many countries [9, 10]. *Brucella abortus* strain 19 vaccine, normally applied as a calfhood vaccine, has been used to prevent brucellosis in cattle worldwide [11]. The vaccine, unfortunately, induces serologic titres that interfered with the identification of cattle infected with the field strain of *B. abortus* [12]. The *B. abortus* strain RB51 vaccine which was licensed in 1996 as the official calfhood vaccine for bovine brucellosis by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA), to date is the only commercially available vaccine in the United States of America that does not interfere with routine serological surveillance and is now approved in that country for use in cattle [13]. The serological response elicited in *B. abortus* RB51 vaccinated animals has been established to be undetected by current conventional tests, can be ascertained by the dot-blot assay [14] and RB51-complement fixation test (RB51-CFT) [15]. The vaccine has also been reported to be effective in preventing brucellosis in elks [16, 17].

Trinidad and Tobago was classified “free of bovine brucellosis” by the Office International Des Epizooties (OIE) in 1984 [18] and subsequent reports also showed the country to be free of brucellosis [18, 19]. However, in 1998, following the identification of seropositive animals [20]. Trinidad and Tobago implemented a nationwide serological screening programme for

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brucellosis using the buffered plate agglutination test (BPAT) which identified 56 farms as positive for the disease. The country also adopted a brucellosis-control programme, which incorporated limited vaccination with a commercial *B. abortus* strain RB51 vaccine as well as a test-and-slaughter policy. However, in a vaccination trial utilizing the commercial *B. abortus* strain, RB51 vaccine at the recommended calfhood dose failed to protect water buffalo calves from infection following natural exposure to *Brucella abortus* biovar 1 [21]. A subsequent study also indicated that experimental exposure of vaccinated water buffalo by the subcutaneous route did not prevent infection following exposure to different doses of a local strain of *B. abortus* biovar 1 subcutaneously. In view of the constraints imposed by the ban on importation of the standard strain of *B. abortus* 2308 used in vaccination trials, attempts were made to obtain virulent local strains of *B. abortus* isolated from the brucellosis outbreak in 1998 [22]. Inoculation of cattle and water buffalo calves with a local strain of *B. abortus* biovar 1 by the intra-conjunctival route using a range of doses, including the recommended dose, did not cause seroconversion or clinical disease but inoculated brucellae were recovered at a significantly higher frequency from the prescapular and parotid lymph nodes of cattle than water buffalo [23]. Diptee *et al*. [24] used different doses of a local strain of *B. abortus* biovar 1 to infect vaccinated and non-vaccinated water buffalo which also failed to induce infection by the intra-conjunctival route, but subcutaneous route resulted in serological and bacteriological evidence of infection but no clinical manifestation. Furthermore, studies on 19 local strains of *B. abortus* biovar 1 to assess their virulence in BALB/c mice indicated that those that originated from water buffalo origin were significantly less virulent than strains recovered from cattle and variability in the pathogenicity of the strains was demonstrated [25]. The study also demonstrated the low virulence of the local *B. abortus* strains and that water buffalo may be more resistant to the pathogen than cattle.

The current study was therefore conducted to determine the efficacy of the RB51 vaccine in water buffalo calves vaccinated at 6-10 months of age using the recommended dose (RD) and challenged intravenously by a local strain of *B. abortus* biovar 1 (Trinidad 1) when they were approximately 6 months pregnant. The study also assessed clinical, serological, bacteriological and pathological changes in vaccinates and non-vaccinates (control), pre- and post-challenge.

MATERIALS AND METHODS

Experimental Groups and Vaccination

The experimental groups, vaccination regimen, and the facilities where the study was conducted on water buffalo calves aged 7 – 10 months were earlier described [26]. Briefly, the calves were randomly divided into three groups with stratification according to size. *Brucella abortus* SRB51 vaccine (Professional Biological Company, Denver, Colorado, USA) was used in this study. Animals from Groups I, II and III at 6 to 10 months of age received the recommended standard dose of RB51 twice four weeks apart (12 animals), the recommended standard dose of RB51 twice 18 weeks apart (12 animals) and phosphate buffered saline (control, 8 animals), respectively. Study animals were housed together in a brucellosis-free quarantine facility until the end of that phase of the study.

Pasture Breeding of Heifers

All heifers were placed at a high plane of nutrition three months subsequent to unsuccessful synchronized artificial insemination. Additional high-quality forage, concentrate, minerals, and vitamins were added and supervised in the intensive system the study animals were kept, for the subsequent 5-6 months.

Two seronegative bulls aged 4-6 years old were introduced to the heifers following the 5-6 month nutritional boost and when the heifers were 27 to 33 months old. Pregnancy diagnosis by rectal palpation was performed on all animals 60 days after the introduction of the bulls and the procedure done monthly until 10 months post-exposure to the bulls.

Determination of Intravenous Dosage of Strain *B. abortus* Trinidad 1 to Induce Clinical Brucellosis

As previous studies reported that the intra-conjunctival and subcutaneous routes of inoculation of water buffalo with local strains of *B. abortus* failed to induce clinical brucellosis [23, 24] an attempt was made to increase the virulence and pathogenicity of a local strain of *B. abortus* Trinidad 1 by passage through pregnant water buffalo by the intravenous route. Two brucellosis-free water buffalo, approximately 6 months pregnant, as assessed by rectal palpation, were housed in a fenced confinement for the study period. The study animals were allowed to acclimatize in the confinement for a period of one week prior to the experimental challenge.

Strain *B. abortus* Trinidad 1, isolated from skin lesions in water buffalo was grown as reported earlier [24]. Both pregnant water buffalo were inoculated intravenously with the cell suspension of strain *B. abortus* Trinidad 1 in phosphate buffered saline (PBS) using the following doses, 2.5 x 10^9 CFU and 1.0 x 10^{10} CFU respectively by the procedure earlier described [24] to determine the mean concentration of brucellae in the cell suspension administered. Clotted blood was collected from the jugular vein of both animals (WB#1 and WB#2) at the following interval: Day-7 (pre-exposure), week 0 (day of exposure), 2, 4, 6 and 8 PIW (post-inoculation week) and at slaughter. The BPAT was used to screen all sera collected for antibodies to *B. abortus*. Both water buffaloes were monitored daily for a total of 8 weeks post-challenge for evidence of abortion and other clinical signs, or delivery of normal calves. On termination of the study, the dams were slaughtered and the following tissues were collected for bacteriological study: placentomes, internal iliac lymph nodes, supramammary lymph nodes, mammary glands, bronchial lymph nodes, retropharyngeal nodes and the parotid lymph nodes. The procedure for the isolation of *B. abortus* biovar 1 without the use of rifampicin (10 µg/ml) was used as earlier described [22].

Experimental Challenge of Pregnant Water Buffalo Heifers by Intravenous Route and Serological Assay

Brucella abortus strain Trinidad 1 that was intravenously passaged through both pregnant water buffaloes above and recovered from uterine tissues and lymph nodes was used to challenge the vaccinated and non-vaccinated pregnant heifers. The protocol used for the growth of the culture and preparation for inoculation of water buffalo was earlier described [24].

All study animals (Groups I, II and III) were inoculated with approximately 1.0 x 10^8 CFU by the intravenous route at approximately 6 months of pregnancy (Table **1**). Thereafter, the dams were placed in confinement to facilitate frequent observation several times daily by farm personnel for evidence of stillbirth, abortion, parturition in progress, normal delivery and conditions of the calves delivered.

The serological status of water buffalo pre- and post-challenge was determined by initially collecting unclotted blood from all study animals at the following intervals: day-3 (pre-challenge), day 0 (day of challenge), and at day 2, day 4; week 1, 2, 4, 6, 8, 10 post-challenge week (PCW). The BPAT was used to

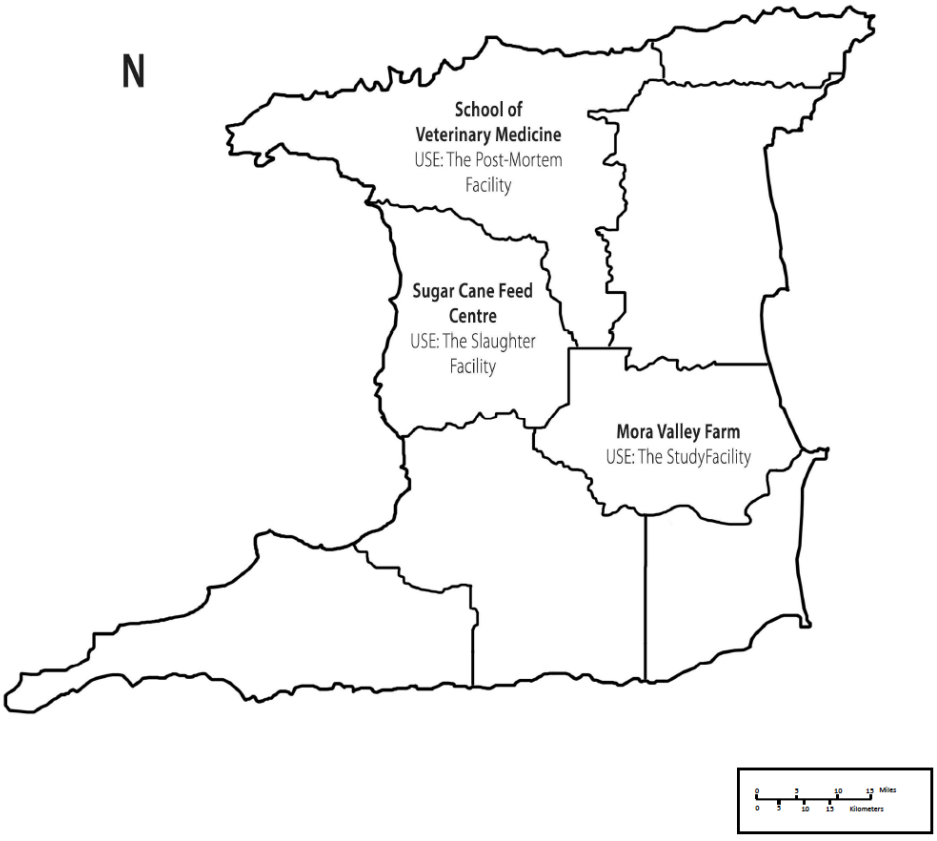

Figure 1: Map of Trinidad highlighting locations used for the study.

screen all sera for antibodies against strain *B. abortus* Trinidad 1.

At abortion or parturition, un-clotted blood was collected from all dams on the same day. The dams were then transported for slaughter at the only government-abattoir (Sugar Cane Feed Centre) for the slaughter of brucellosis-positive animals, located at approximately 80 km from the farm where the study was conducted (Figure **1**). Un-clotted blood was also taken from all calves born alive on the day of parturition and then again just prior to euthanasia for use to screen for *B. abortus* antibodies by the buffered plate agglutination test (BPAT) using United States Department of Agriculture (USDA) S1119.3 antigens and established protocol [27]. Any degree of visible agglutination on the BPAT was considered a positive reaction [27, 28].

Post-Challenge Outcome

Live calves were transported to the UWI-SVM necropsy facility for blood collection, euthanasia and post-mortem examination by specialist veterinary pathologists. Foetuses or dead calves were double-bagged and put in cooler with ice for immediate transportation to the post-mortem facility at the UWI-SVM. The number of days post-challenge when abortion or parturition occurred was recorded.

For the dams, post-mortem examinations were conducted within 72 h of parturition/ abortion of calves and their status classified as full grown/healthy or premature and born dead, born weak and /died or euthanized based on a condition score and physical examination. The sex of each calf/fetus was noted.

A detailed examination of the following systems in both calves and dams was undertaken: Integumentary/ External, Internal (General), Digestive, Cardiovascular, Haemopoietic, Respiratory, Musculoskeletal, Urinary, Endocrine, and Reproductive. Calves submitted dead were also checked to determine if they were born dead or alive by the ability of their lungs to float in water.

Recovery of *Brucella abortus* from Tissues of Dams, Aborted Foetuses, and Normal Calves

The tissues collected for the bacteriological study were whole blood, uterus (swab), supra-mammary lymph nodes, liver (with lymph nodes), lungs (with lymph nodes), kidney (with renal lymph nodes), internal and external iliac lymph nodes, mesenteric lymph nodes, heart, and spleen. Excess fat was trimmed off on site and tissues placed individually into sterile plastic Whirl-Pack® bags (Nasco, Fort Atkinson, Wisconsin, USA), sealed and transported ice-cooled to the laboratory within 1-2 h for processing immediately or frozen at -20ºC until processed, depending on time of day samples arrived. The tissues collected from calves were rectal swabs, abomasal swabs, liver, heart, lungs, mesenteric lymph nodes, spleen and kidney which were handled the same way as samples from the dams.

Processing and culture of tissues for isolation of *B. abortus* were performed in a class II biohazard cabinet using the procedure earlier described for bacteriological culture [22, 24]. All suspect isolates of *B. abortus* were tentatively identified using standard methods and stored in skimmed milk in 1 ml aliquots at -40ºC until sent to the Central Veterinary Laboratory, Weybridge, UK, a brucellosis reference laboratory, for confirmation as *B. abortus* biovar 1.

Data Analysis

Data were analysed with a one-way analysis of variance (ANOVA), factorial ANOVA and correlation analysis using a general linear model. One way ANOVA was used to investigate significant treatment effects in the following situations, the independent variable being the treatment group and the dependent (response) variables were the post-challenge period for abortion/delivery, and isolation of *B. abortus* biovar 1 from study animals. Statistically significant effects were subsequently subjected to follow-up analyses using Dunnett's t-tests. The Dunnett's t-test was used as the multiple comparison techniques when comparisons were made with vaccination groups and the control group. Factorial analysis of variance was used to investigate interactions between treatment groups and the frequency of isolation of brucellae from selected tissues. Specificity and sensitivity were determined as described [29, 30]. An independent samples t-test was used to compare the sensitivity for tissue of dams and calves in detecting *B. abortus* biovar 1. A priori, it was agreed that all statistical significance tests conducted would be two-tailed and interpreted at an alpha level of 0.05. Statistical Package for Social Sciences (SPSS) version 9.0 was used to manage and run the statistical data analyses.

Approval by Ethics Committee

The Ethics Committee of the Faculty of Medical Sciences of the University of the West Indies approved

of the research protocol and monitored the care of the experimental animals.

RESULTS

Pregnancy Following Natural Breeding

Following natural service of the heifers, a total of 21 pregnant heifers were considered appropriate (similar stage of pregnancy) and available for the experimental challenge. Of these pregnant heifers, 9, 7 and 5 belonged to Groups I, II and III (control) respectively. However, because heat detection, AI, and pregnancy were not synchronized, the stage of pregnancy was slightly variable in the pregnant heifers to be eligible for the challenge (at approximately 6 months) and therefore the pregnant heifers were grouped for a challenge in a staggered fashion.

Determination of Intravenous Dosage of *B. abortus* Trinidad 1 to Elicit Brucellosis in Two Pregnant Water Buffalo

The two serologically negative pregnant water buffalo, WB # 1 and WB # 2, seroconverted at 2 post-inoculation weeks (PIW) of intravenous injection. At 7 PIW, WB #2 aborted a very weak calf (approximately 11 weeks premature) which died shortly after birth while at 8 PIW, WB # 1 aborted a live offspring (approximately 10 weeks premature) which died on the same day. All samples tested for *B. abortus* biovar 1 from WB # 2 were bacteriologically negative for the organism while WB #1 at slaughter was seropositive (3+) by the BPAT. *B*. *abortus* biovar 1 was recovered from the uterus and its associated lymph nodes, and this isolate was used as the challenge inoculum in the 21 pregnant water buffalo (Groups I, II and III) in the current study.

Experimental Challenge of Pregnant Water Buffalo with *B. abortus* Biovar 1: Serological Status

The mean ± sd interval in months between the administration of the booster dose of RB51 vaccine and experimental challenge in the current study was 31.3 ± 2.75, 27.0 ± 2.83 and 31.3 ± 2.52 for Groups I, II and III animals respectively (Table **1**).

All pregnant water buffalo were serologically negative on BPAT prior to challenge and on the day of challenge week 0, i.e. day of challenge (PCW 0). For the three Groups (I, II and III), by day 2 post-challenge, 76.2% (16/21) of the study animals seroconverted and by post-challenge week 2 (PCW 2), the highest frequency of seropositive water buffalo, 95.2% (20/21) was achieved. By the end of sequential sampling (PCW 10), the seropositive rate was reduced to 90.5% (19/21) because 1 earlier seropositive cow (Group II) had become seronegative. Overall, by the time the dams experienced normal parturition or abortion, the seropositive rate was 76.2% (16/21).

A comparison of the study groups revealed respectively for Groups 1, II and III that by Day 2 post-challenge, the rate of seroconversion was 87.5% (7/8), 85.7% (6/7) and 60.0% (3/5); by PCW 10, 100.0% (9/9), 71.4% (5/7) and 100.0% (5/5) (Table **1**). The differences were not statistically significant (P>0.05). The rate of seroconversion was similar for Groups I and III from PCW 2 to 10 with all animals seropositive (100.0%). The rate of change from seropositive status at PCW 10 to the seronegative status at normal parturition or abortion was 11.1% (1/9), 28.6% (2/7) and 40.0% (2/5) for Groups I, II and III respectively. The Group I animal was seropositive (2+ and 3+) in PCW 2, 4, 6, 8 and 10 but became seronegative at parturition/abortion. Both animals in Group II that converted from being seropositive to seronegative had weak BPAT reactions (1+) between PCW 1 - 4. Reactions for both seronegative animals in Group III were strong (3+) in PCW 4, 6, 8 and 10.

Post-Challenge Outcome

Of the 9 dams in Group I, 5 (55.6%) delivered dead calves/weak calves/early neonatal deaths compared with 3 (42.9%) out of 7 and 2 (40.0%) out of 5 dams in Groups II and III, respectively. The differences were not statistically significant (P>0.05).

The frequency of abortion/stillbirth/early neonatal death for all seropositive dams i.e. infected with *B. abortus* biovar 1, was 62.5% (10/16); the frequency amongst Groups I, II and III seropositive dams was 62.5% (5/8), 60.0% (3/5) and 66.7% (2/3), respectively (P>0.05).

Amongst the 16 seropositive dams, the mean ± sd number of post-challenge days was 103.6 ± 33.0 (all deliveries) (p=0.067), 92.1 ± 22.0 (dams that experienced normal parturition/aborted/stillbirths/early neonatal deaths (p=0.052) and 79.6 ± 14.0 (dams with abnormal deliveries, i.e. abortion/stillbirths/early neonatal deaths) (p=0.081). The corresponding values were 111.8 ± 31.4, 115 ± 31.4 and 94.0 ±12.6 for Group I dams, 98.1 ± 29.6, 83.8 ± 12.2 and 77.3 ± 9.3 for Group II dams and 101 ± 36.5, 77.0 ± 20.7 and

Table 1: Serological Responses of Pregnant Water Buffalo (*Bubalus bubalis*) Challenged Intravenously with *B. abortus* Biovar 1

Experimenta group	Numbet of animals	Estimated stage of pregnancy (days)	Mean (± sd) interval (months) between booster* vaccination and experimental challenge	Mean dose of *B. abortus* biovar 1 (cfu)	Serological status pre-challenge (BPAT)** Day – 3	No. (%) with serological status post-challenge week (PCW) using the BPAT:									Serological status at delivery/ abortion
						Day			Week						
						0	2	4	1	2	4	6	8	10	
I	9	176	31.3 ± 2.75	3.3×10^8	0 (0.0)	0 (0.0)	7 (87.5)	7 (87.5)	7 (87.5)	9 (100.0)	9 (100.0)	9 (100.0)	9 (100.0)	9 (100.0)	8 (88.9)
II	7	179	27.0 ± 2.83	3.5×10^8	0 (0.0)	0 (0.0)	6 (85.7)	6 (85.7)	6 (85.7)	6 (85.7)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)
III	5	179	31.3 ± 2.52	3.2×10^8	0 (0.0)	0 (0.0)	3 (60.0)	3 (60.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	3 (60.0)
Total	21	178	NA	3.3×10^8	0 (0.0)	0 (0.0)	16 (76.2)	16 (76.2)	18 (85.7)	20 (95.2)	19 (90.5)	19 (90.5)	19 (90.5)	19 (90.5)	16 (76.2)

*Vaccine booster administered to Groups I and II calves at 4 weeks and 18 weeks respectively.
**BPAT results were read as 1+, 2+, 3+ or 4+ but any definite agglutination was classified as a positive response.
NA-Not Applicable.

Table 2: Gross Pathological Findings in the Systems of Calves Delivered by Dams in the Three Experimental Groups

Experimental group	Number of animals	Mean (± sd) interval (months) between booster* vaccination and experimental challenge	No. (%) that are:		[a]Autolysis:			Status of calf: No. (%) in:		No. (%) in the categories:							
										Status on Intergumentary/External:		Internal/Body condition		Respiratory tract	Musculoskeletal		
			Male	Female	0	1	2	Fully grown	Premature	Full hair coat	Hairless/thin hair coat	Good	Fair	LF***	LS***	NAD	Dry muzzle
I	9	31.3 ± 2.75	2 (22.2)	7 (77.8)	4 (44.4)	4 (44.4)	1 (11.1)	4 (44.4)	5 (55.6)	2 (22.2)	7 (77.8)*	7 (77.8)	2 (22.2)	6 (66.7)****	3 (33.30	7 (77.8)	2 (22.2)
II	7	27.0 ± 2.83	3 (42.9)	4 (57.1)	6 (85.7)	1 (14.3)	0 (0.0)	4 (57.1)	3 (42.9)	0 (0.0)	7 (100.0)	6 (85.7)	1 (14.3)	4 (57.1)****	3 (42.9)	6 (85.7)	1 (14.3)
III	5	31.3 ± 2.52	3 (60.0)	2 (40.0)	4 (80.0)	0 (0.0)	1 (20.0)	3 (60.0)	2 (40.0)	0 (0.0)	5 (100.0)**	4 (80.0)	1 (20.0)	3 (60.0)****	2 (40.0)*****	3 (60.0)	2 (40.0)
Total	21	NA	8 (38.1)	13 (61.9)	14 (66.7)	5 (23.8)	2 (9.5)	11 (52.4)	10 (47.6)	2 (9.5)	19 (90.5)	17 (81.0)	4 (19.0)	13 (61.9)	8 (38.1)	16 (76.2)	5 (23.8)

LF--Lung floated.
LS--Lung sank.
NAD-No abnormality detected.
Digestive tract--NAD for Groups I and II but ; 1 animal in Group I had Toxocara spp. +++.
*Two were dehydrated.
**Two were moderately dehydrated.
***LF--Lung floated in water, LS-Lung sank in water.
****LF: 1 lung in Group I with pulmonary oedema;2 lungs with pulmonary oedema in Group II; in Group III: LF-2 with pulmonary oedema.
*****LS: Group III-1 with continuous amniotic fluid.
[a]The degree of autolysis was noted as: 0 – no autolysis.
1 – mild / moderate autolysis.
2 – significant autolysis.
3 – advanced autolysis.

67.5 ± 17.7 for Group III dams. The differences were however not statistically significant (P>0.05).

Characteristics and Findings in Calves at Post-Mortem

Of a total of 21 calves (13 female and 8 male) delivered by the dams, 8 (38.1%) arrived at the post-mortem room dead (Group I=3, Group II=3, and Group III=2), and therefore could not be serologically tested. For the serological status of both the dams and calves, there was 100.0% agreement (i.e. both dam and calf having the same status either positive or negative). Overall, across the three groups, 8 (100.0%) of 8 animals had dam seropositive/calf seropositive results and 5 (100.0%) of 5 had dam seronegative/calf seronegative test results.

Table **2** displays the pathological lesions detected during the post-mortem examination of the calves delivered by dams in the three groups. For Group I animals, 4 (44.4%) of 9 calves were categorized as full-grown or within 2 weeks of normal parturition based on physical characteristics; 5 (55.6%) out of 9 were classified as premature and 5 (62.5%) out of 8 calves were from seropositive dams at parturition/abortion were premature.

In Group II animals, of the 7 calves, 4 (57.1%) and 3 (42.9%) were determined to be full grown and premature respectively. Three (60.0%) of 5 calves were determined to be premature for seropositive dams.

In Group III animals, of the 5 calves, 3 (60.0%) and 2 (40.0%) were full grown and premature respectively on delivery. Two (66.7%) of 3 calves from seropositive dams were premature.

All 5 (100.0%) calves from seronegative dams in the three study groups were classified as full-grown.

The degree of autolysis at post-mortem was generally low with 66.7% (14/21) of the animals showing no signs of autolysis (Grade 0). Five (23.8%) and 2 (9.5%) animals displayed signs of grade 2 and grade 3 autolysis respectively. In all 11 (100%) calves, classified as full-grown, the lungs floated as was similarly found in 2 (20%) of 10 calves classified as premature, thus indicating that these 2 calves were born alive.

In 6 calves, a gross diagnosis of pulmonary oedema was made due to fluid/froth accumulation in the bronchi and trachea. There were no abnormalities detected grossly in any calf at post-mortem in the reproductive, urinary, haemopoetic, liver and cardiovascular organs. In one calf numerous adults of *Toxocara vitulorum* were present in the small intestines. In 19.0% (4/21) of the calves, mild to moderate level of dehydration was observed at post-mortem on the observation of very dry skeletal muscles.

Recovery of *Brucella abortus* from Calf Tissues

Tissue samples for 5 (100.0%) of 5 calves (Groups I -1, II - 2 and III - 2) from all seronegative dams at delivery were bacteriologically negative for *B. abortus,* which yields a specificity of 100% using the 8 tissues/sample types studies (Table **3**).

Brucella abortus biovar I was recovered most frequently, 100.0% (8/8) from rectal swabs and abomasal samples of seropositive calves compared with the other 6 tissues/organs sampled from Group I calves. Amongst Group II calves, the isolation rate of *B. abortus* was 80.0% (4/5) of *B. abortus* biovar 1 from each tissue sampled (rectal swabs, abomasal swabs, lung tissues, mesenteric lymph nodes, spleen, kidney, liver, and heart). *Brucella abortus* biovar 1 was isolated at a rate of 100.0% (3/3) from six tissues/organs with the liver (50.0%) and heart tissues (33.3%) being the exceptions.

For samples cultured from seropositive premature calves, in Group I, in 4 (80.0%) out of the 5 calves the frequency of isolation of *B. abortus* biovar 1 from the tissues/organs tested was 100.0% (8/8); in the 2 (66.7%) out of 3 seropositive premature calves all (100.0%) 8 tissues were positive for *B. abortus* biovar 1 but in 1 calf none (0.0%) of the 8 tissues yielded the pathogen; amongst the 2 seropositive premature Group III calves, the isolation rate from the 8 tissues were 87.5% and 100.0%.

Amongst 3 Group I seropositive, full-grown calves, the isolation rates for *B. abortus* biovar 1 for the 8 tissues tested ranged from 37.5% to 100.0%; for the two full grown seropositive calves in Group II, the isolation rate was 100.0% from the 8 tissues compared with a rate of 75.5% in the only Group III calf.

The overall sensitivity for detection of *B. abortus* biovar 1 from Group I animal samples was 79.7% (51/64); for Group II animal samples, 80.0% (32/40) and for Group III animal samples 85.0% (17/20). Overall, there was no statistically significant difference in the frequency of isolation of *B. abortus* biovar 1 within groups (p = 0.935).

Table 3: Frequency of Isolation of *B. abortus* Biovar 1 from Tissues of Calves in the Three Groups

Tissue cultured for *B. abortus* biovar 1	Experimental Group I		Experimental Group II		Experimental Group III	
	No. (%) culture-positive amongst:		No. (%) culture-positive amongst:		No. (%) culture-positive amongst:	
	All calves (n=9)	Seropositive calves (n=8)	All calves (n=7)	Seropositive calves (n=5)	All calves (n=5)	Seropositive calves (n=3)
Rectal swab	8 (88.9)	8 (100.0)	4 (57.1)	4 (80.0)	3 (60.0)	3 (100.0)
Abomasal swab	8 (88.9)	8 (100.0)	4 (57.1)	4 (80.0)	2 (50.0)**	2 (100.0)**
Liver tissue	5 (55.6)	5 (62.5)	4 (57.1)	4 (80.0)	1 (25.0)**	1 (50.0)**
Kidney tissue	5 (55.6)	5 (62.5)	4 (57.1)	4 (80.0)	3 (60.0)	3 (100.0)
Lung tissue	7 (77.8)	7 (87.5)	4 (57.1)	4 (80.0)	3 (60.0)	3 (100.0)
Mesenteric lymph node	7 (77.8)	7 (87.5)	4 (57.1)	4 (80.0)	2 (50.0)*	2 (100.0)*
Heart tissue	5 (55.6)	5 (62.5)	4 (57.1)	4 (80.0)	1 (20.0)	1 (33.3)
Spleen tissue	6 (66.7)	6 (75.0)	4 (57.1)	4 (80.0)	2 (50.0)*	2 (100.0)*

*Based only on samples tested.

Overall, for the three groups, the sensitivity for using rectal and abomasal swabs was comparatively high for the isolation of *B. abortus* biovar 1, 93.8% (15/16) and 93.3% (14/15). The differences in the frequency of isolation across type of tissues for the groups were not statistically significantly different (P>0.05).

Recovery of *Brucella abortus* from Dam Tissues

All (100.0%) of the 5 seronegative water buffalo (*Bubalus bubalis*) dams failed to yield *B. abortus* in all the tissues subjected to bacteriological analysis. Therefore, a specificity of 100% was calculated for *Brucella* isolation using the specified media used for isolation (Table **4**).

B. abortus biovar 1 was recovered most frequently from the supramammary lymph nodes, uterine swabs and internal/external iliac lymph nodes of Group I animals at a frequency of 100.0% (8/8), 87.5% (7/8) and 75.0% (6/8) respectively. For Group II animals, *B. abortus* was recovered at 80.0% (4/5), 80.0% (4/5) and 60.0% (3/5) for the supramammary lymph nodes, uterine swabs, and internal/external iliac lymph nodes respectively. For Group III animals, the highest rate of recovery was also from the supramammary lymph nodes, followed by uterine swabs and the internal /external iliac lymph nodes at 100.0% (3/3), 66.7% (2/3) and 100.0% (3/3) respectively.

It was least likely (0.0%) to isolate *B. abortus* biovar 1 from the spleens of seropositive animals in Group I animals, mesenteric lymph nodes in Group II and the kidneys, lungs, mesenteric lymph nodes, heart and spleens in Group III.

The overall sensitivity, using the 8 tissues from seropositive dams, to isolate *B. abortus* biovar 1 was 40.6% (26/64), 42.5% (17/40) and 33.3% (8/24) for Groups I, II and III dams respectively. Overall, there was no statistical difference in the frequency of isolation of *B. abortus* biovar 1 within groups (p = 0.745).

For the 3 groups, the sensitivity for the type of tissues used for detecting *B. abortus* biovar 1 was statistically significantly higher in supramammary lymph nodes, 93.8% (15/16), uterine swabs, 81.3% (13/16) and the internal/external iliac nodes, 75.0% (12/16) compared with the remaining five tissues where the rates were less than 20.0%. The differences were statistically significant (P<0.05).

Comparison of Sensitivity of Calf Samples and Dam Samples to Detect *B. abortus* Biovar 1

An independent t-test was used to compare the sensitivity of calf and dam samples to detect *B. abortus* from experimentally challenged pregnant water buffalo. Amongst seropositive dams and their corresponding seropositive calves, the frequency of isolation of *B. abortus* biovar 1 from the 8 tissues (supramammary lymph nodes, uterine swab, internal/external iliac lymph nodes, kidney, lungs, mesenteric lymph nodes, heart, spleen) regarded to have the highest rate of isolation, was significantly higher (p=0.000) in calves (81.3%) than in dams (39.8%) (data not shown).

A comparison of the frequency of isolation *B. abortus* biovar 1 from five tissues (heart, spleen, mesenteric lymph nodes, kidney, and lungs) common to both the seropositive dams and corresponding

Table 4: Frequency of Isolation of *B. abortus* Biovar 1 from Tissues of Dams of the Three Groups

Tissue cultured for *B. abortus* biovar 1	Experimental Group I		Experimental Group II		Experimental Group III	
	No. (%) culture-positive amongst:		No. (%) culture-positive amongst:		No. (%) culture-positive amongst:	
	All dams (n=9)	Seropositive dams (n=8)	All dams (n=7)	Seropositive dams (n=5)	All dams (n=5)	Seropositive dams (n=3)
Supramammary lymph node	8 (88.9)	8 (100.0)	4 (57.1)	4 (80.0)	3 (60.0)	3 (100.0)
Uterine swab	7 (77.8)	7 (87.5)	4 (57.1)	4 (80.0)	2 (40.0)	2 (66.7)
[a]Int. & Ext. Iliac lymph node	6 (66.7)	6 (75.0)	3 (42.9)	3 (60.0)	3 (60.0)	3 (100.0)
Kidney plus lymph node	1 (11.1)	1 (12.5)	2 (28.6)	2 (40.0)	0 (0.0)	0 (0.0)
Lung plus lymph node	1 (11.1)	1 (12.5)	1 (14.3)	1 (20.0)	0 (0.0)	0 (0.0)
Mesenteric lymph node	2 (22.2)	2 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Heart tissue	1 (11.1)	1 (12.5)	1 (14.3)	1 (20.0)	0 (0.0)	0 (0.0)
Spleen tissue	0 (0.0)	0 (0.0)	2 (28.6)	2 (40.0)	0 (0.0)	0 (0.0)

[a]Internal and External iliac nodes.

seropositive calves demonstrated statistically significant (p=0.000) higher proportion of calf tissues (78.8%) yielded the organism than dam tissues (17.5%) (data not shown).

For the two tissue samples with the highest rate of recovery of *B. abortus* biovar 1 for seropositive dams (supramammary lymph node and the uterine swab) and for seropositive calves (abomasal swab and rectal swab), the frequency of isolation of *B. abortus* was 87.5% and 93.8% respectively but the difference was not statistically significant (p=0.518).

Maternal and Foetal Infection Rate

Maternal or foetal infection rate, defined as the recovery of the *B. abortus* biovar 1 strain from any maternal or foetal sample, was calculated to be 100.0% (8/8) for Group I animals, 80.0% (4/5) for Group II and for Group III (control) animals, 100.0% (3/3) (data not shown).

DISCUSSION

The preliminary passage of *B. abortus* Trinidad 1 in the two pregnant water buffalo successfully increased its infectivity and virulence by being able to induce abortion at a dosage of 10^9 cfu by the intravenous route in vaccinated and non-vaccinated water buffalo in the current study. This was demonstrated by the fact that after intravenous challenge with *B. abortus* Trinidad 1, all animals seroconverted by PCW 2 and on at least two consecutive occasions by the BPAT. These findings were not unexpected since the O-side chain is the main antigen in smooth brucellae. Data generated in the current study suggest that strain RB51-vaccinated water buffalo that were subsequently challenged by a field strain of *B. abortus* biovar 1, developed antibodies that were detected by the standard serologic tests such as the BPAT. The challenge strain *B. abortus* biovar 1 was isolated from several tissues from both maternal and foetal sources at necropsy. Importantly, colonization, measured as the number of *B. abortus* positive samples per heifer, was not statistically different in vaccinated groups compared to the control group for this study (p=0.745 – dam; p=0.935 – calf/foetus). This result further suggests that the RB51 vaccine did not protect against infection in water buffalo. In a challenge study in cattle [32], it was reported that colonization, as measured by the number *B. abortus* positive samples per heifer, was more intense in non-vaccinated group when compared to the vaccinated group (p=0.04), an expected finding since the RB51 vaccine has been established to prevent infection and abortion by *B. abortus* in cattle [31, 32]. This is an indication that the vaccine did not prevent infection in water buffalo by a field strain of *Brucella abortus*.

It is of interest to note that 5 (23.8%) out of the 21 water buffalo that seroconverted following intravenous challenge by *B. abortus* biovar 1 became seronegative by the time of normal parturition or abortion. This finding did not appear to be treatment group-related since they were detected in Groups I, II and III. These results can be attributed, in part, to the fact that BPAT is a qualitative agglutination test and when the degree of agglutination is weak, the results between testing in

the same animal may be subjective and variable. In a study in American bison (*Bison bison*), the serological titres, as determined by the STAT, were virtually undetectable, post-challenge (*B. abortus* 2308) for non-aborting animals at the time of necropsy [33]. This is in agreement with the findings in the current study where all seronegative water buffalo did not abort and delivered full term normal calves. Another explanation may be that those animals were possibly naturally more resistant due to the presence of the NRAMP1 gene [34] and hence cleared the organism at a faster rate.

RB51 vaccination using the recommended dose in this study did not confer protection against infection and abortion for domestic water buffalo (*Bubalus bubalis*) under experimental challenge conditions. It is well established in the literature that protection against brucellosis is measured by a significant decrease in abortions or birth of weak calves, and a significant decrease in *B. abortus* colonization of tissues of vaccinated cattle when compared to non-vaccinated controls after challenge [35]. Therefore, the isolation of the challenge strain from different tissues of the aborting dams and the fetuses was used as a benchmark for brucellosis infection, as it is widely recognized to be the most definitive criterion for measuring the effect of brucellosis vaccination [32]. Furthermore, the fact that there was no statistically significant difference in the rate of infection and abortion between vaccinated and non-vaccinated water buffalo is evidence that RB51 vaccine did not protect vaccinated animals against brucellosis when experimentally challenged.

In this study, the RB51 vaccine administered at the recommended dose protected 37.5% and 40.0% of water buffalo against abortion in Groups I and II animals respectively, while 33.3% of animals in Group III (unvaccinated controls) did not abort following experimental challenge. Furthermore, protection against infection (based on serological findings) by the challenge *B. abortus* strain was 0.0% and 20.0% for dams and calves/foetuses respectively for animals in both vaccinated groups (I and II), while 0.0% for animals in control group were protected from infection. These findings in water buffalo vaccinated with RB51 are considerably lower than the protection against infection in dams and calves/foetuses reported for vaccinated cattle (65.0% and 87.0%, respectively) [31, 32]. A study in American bison reported a 22.0% protection against infection in dams vaccinated with RB51, however, in the same study, protection against foetal infection in RB51-vaccinated bison was 81.0% [33] compared to 0 – 20.0% in our study. The 100.0% infection rate in the control group was higher than that reported for a similar study in cattle (60.0%) [31], but this was not statistically significantly higher than our infection rate in vaccinated dams (80.0 – 100%).

The rather low protection rate (37.5 – 40.0%) against abortion following administration of RB51 vaccine achieved in the current study is considerably lower than what has been reported for similar studies in cattle (88.8%, 75.0%) [32, 36] and bison (85.0%) [33], coupled with similarity in the infection and abortion rates amongst vaccinated and non-vaccinated controls, clearly indicate that the RB51 vaccine in water buffalo was ineffective in providing protection in challenged pregnant animals, contrary to what was reported for cattle and bison challenged with *B. abortus* S2308 [32, 33, 36]. The findings in the current study is however in agreement with reports of non-protection by RB51 vaccine in preventing infection and abortion in elk (*Cervus elaphus*) using the recommended calfhood dose followed by a booster of the same, one year later [16, 17], suggesting that breed variation may influence the effectiveness of RB51 vaccine. It was also demonstrated earlier in Trinidad and Tobago that RB51 vaccine failed to prevent infection in vaccinated water buffalo calves that were naturally exposed to *B. abortus* biovar 1 [21]. It is, however, pertinent to mention that Caporale *et al*. [37] in a preliminary study using RB51 vaccine at 3 times greater the dose recommended in cattle for water buffalo in Italy reported that RB51 vaccine may have offered some protection to calves challenged with virulent *B. abortus* strain 544. The low sample size (5) of water buffalo used in that study however limited any conclusive evidence of the vaccine's effectiveness and the authors suggested a future study using more animals.

Considering the relatively high rate of infection and abortion detected in this study amongst both vaccinated and unvaccinated animals, it is appropriate to consider the possible effects of both the dose and route of administration of the challenge strain of *B. abortus* Trinidad 1. The challenge dose of *B. abortus* biovar 1 used in the current study was in the range 2.5 – 4.4 x 10^8 CFU which is 20 – 40 times the recommended standard challenge of 1 x 10^7 in cattle and 10 times the dose reported for bison [33]. The higher dose used in the current study was based on the doses determined in our preliminary study which caused abortion in two pregnant water buffalo. The possibility that the high dosage used may have

overwhelmed the protection offered by an RB51 vaccine can therefore not be ignored. It has been suggested in studies in cattle and bison that vaccine-induced protection can be overwhelmed if challenge dosages are too high [38, 39]. Data obtained in the current study, however, suggest that an overwhelmed host immune system may not be the primary factor for the abortion episodes observed. This is based on the fact that, abortions occurred in our study between 9 (63 days) and 14 weeks (98 days) post-challenge in seropositive dams, which is the normal range for abortion to occur in animals when the immune system was not overwhelmed [32, 33, 36]. Secondly, the times of induction of abortions in water buffalo in the current study occurred much later than that reported for cattle, also challenged by the IV route, using different *B. abortus* strains. In fact, S19 induced abortion in 100% of the animals between 16 and 42 days post-challenge, *B. abortus* 45/20 caused abortions (100%) between 9 and 47 days post-challenge and *B. abortus* strain 544 resulted in abortion in all (100%) between 3 – 4 weeks [40]. It has been reported in bison subsequent to intra-conjunctival (IC) challenge with 10^7 CFU of S2308 that abortions (62.0% non-vaccinated, 15.0% vaccinated) occurred in 5 – 8 weeks post-challenge [33].

The failure to isolate *B. abortus* biovar 1 from any tissues of seronegative water buffalo at the time of delivery of their calves was not unexpected, since isolation of the challenge strain from tissue samples is considered an indication of brucellosis infection [32] and the five seronegative animals would have cleared any infection due to challenge prior to parturition. The challenge strain was recovered from both aborting and non-aborting dams and from calves of dams as long as the dams were serologically positive. This is consistent with challenge studies conducted in cattle [32] and the American bison [33]. It was however reported that seropositive bison did not abort and the recovery of challenge strain was low in maternal lymphatic tissue [33]. The current study suggests that the supramammary lymph node of the dam is a good site for the recovery of *B. abortus* in water buffalo with a sensitivity of 93.8% for both seropositive aborting and non-aborting dams. The supramammary lymph nodes have been reported to be good sources of brucellae in dams of cattle as well [32].

In both vaccinated groups and the control group, the frequency of isolation of *B. abortus* was higher in uterine swabs and supramammary lymph nodes, and the internal/external iliac lymph nodes (>75.0%) whereas in aborted foetuses, premature weak calves or infected live calves, higher frequency isolation occurred in abomasal swab, rectal swab, lungs, mesenteric lymph node and spleen (>80.0%). These findings in water buffalo are similar to reports of studies in cattle and the American bison where it was reported that the most frequently infected tissues in *B. abortus* infected animals were placentomes, uterus/vaginal swab, milk of dams, and from lungs, spleen, and rectal swabs of foetuses [32, 33, 41].

In the current study, the finding of a significantly ($p=0.000$) higher rate of recovery of *B. abortus* from calf tissues compared with dam tissues may be explained, in part, by the low (0 – 20%) level RB51-induced protection against foetal infection, similar to the dams, except that the foetus/calf is incapable of mounting a sufficiently competent immune response. It has been reported in cattle that there is a possibility of placental and foetal infection following intravenous administration of SRB51 due to haematogenous spread [40] and that this route of infection may also be relevant in the water buffalo following IV administration of *B. abortus* biovar 1. The RB51 vaccine strain was not recovered at any time from maternal or foetal samples collected at necropsy which occurred 150 – 160 weeks post-vaccination/booster vaccination; a finding in agreement with published reports [32, 33, 36].

Gross pathological findings in the current study were not significantly different between vaccinated and non-vaccinated water buffalo. It was however of interest that pulmonary edema was detected in 23.8% (5/21) of normally delivered full grown calves comprising 2 seropositive and 3 seronegative animals. A study on brucellosis-induced abortion in cattle failed to observe any gross lesions suggestive of typical bronchopneumonia despite the recovery of *B. abortus* from the tissues in aborted foetuses [33] but Davis *et al*. [42] reported that aborted foetuses from non-vaccinated bison following the experimental infected with S2308 showed signs of bronchopneumonia while those from SRB51-vaccinated did not [43]. It is, therefore, possible that pulmonary oedema observed in aborted foetuses in the current study was due to the fact that the RB51 vaccine was ineffective and showed lesions similar to what was reported for challenged unvaccinated bison.

A number of constraints were encountered in the study which contributed to some of its limitations, namely: (a) lack of access to *B. abortus* strain 2308, the standard challenge strain for vaccination trials on

brucellosis; the use of a local strain of *B. abortus* Trinidad 1 makes it difficult to compare results of studies that used different strains; (b) the experimental facility, where the study animals were located, was approximately 100 hundred kilometers away from the laboratory and slaughter facilities thereby posing logistical challenge in transporting inoculum and receiving samples for processing in the laboratory, and the slaughter of animals; (Figure **1**) and finally, (c) the use of natural breeding resulted in a staggered (over 7 – 8 months) pregnancy of water buffalo used in the study which led to the growth of challenge cultures several times. The possibility of variability in determining the stage of pregnancy by rectal examination and culture does therefore exist.

CONCLUSIONS

The present study is the first trial in the country that used the local isolate *B. abortus* biovar 1 to challenge vaccinated and non-vaccinated pregnant water buffalo for evidence of infection and abortion. Although the study was conducted by infecting the animals intravenously, which is not the natural route of infection, it is concluded that the RB51 vaccine at the recommended dose was ineffective in protecting water buffalo.

It is recommended that a local virulent strain of *B. abortus* should be developed or isolated for use in vaccine-challenge trials on brucellosis.

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