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# Lactogenic immunity following vaccination of cattle with bovine coronavirus

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## Abstract

In order to investigate the ability of an oil adjuvanted vaccine containing bovine coronavirus antigen to enhance lactogenic immunity in the calf, pregnant cows and heifers were vaccinated and specific virus neutralising antibody levels determined in serum, colostrum and milk. Pre-existing antibody titres (as a result of natural infection) in the serum of these animals were found to be significantly increased as a result of a single shot vaccination carried out between 2 and 12 weeks before calving. This was reflected in a similar increase in the titre and duration of specific antibody in milk and colostrum that was passed on to the calves. The overall response observed was highly dependent on an adequate antigen payload being incorporated within the single dose vaccine. No abnormal local or systemic reactions were observed as a result of vaccination. It is hoped that this approach will lead to the production of a superior commercial vaccine for the protection of neonatal calves against enteric coronavirus infection. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Bovine coronavirus; Cattle; Lactogenic immunity

## 1. Introduction

Neonatal diarrhoea is a complex disease associated with a number of infectious agents occurring either singly or in combination [1,2,3]. In calves, economic losses are suffered as a result of mortality, which can reach up to 80%, and also veterinary costs and decreased productivity of survivors. The viral agents most commonly associated with this syndrome are rotavirus and coronavirus, both of which have been shown to be primary pathogens in calves [4,5]. Although this syndrome is most overtly associated with young animals, subclinical infections are also common in adults which may therefore act as reservoirs for reinfection [6].

Passive immunity against enteric viral infections is dependent upon the continual presence of a protective level of specific antibody in the gut lumen. This can be achieved by allowing the neonate to ingest colostrum or milk containing these specific antibodies from its dam (lactogenic immunity). Although most adult cattle are seropositive for both rotavirus and coronavirus antibodies [7], during the transition from colostrum to milk production there is a dramatic decline in antibody titres which partially explains the high incidence of infection in calves older than 5 days. Successful rotavirus vaccines aimed at increasing both the titre and duration of specific antibody in both colostrum and milk have been developed [8], however, similar success has not been reported with vaccines targeted against bovine coronavirus [9–13].

This paper reports the ability of a new single dose vaccine to significantly increase both the level and duration of coronavirus neutralising antibodies in the serum and milk of vaccinated cattle.

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## 2. Materials and methods

### 2.1. Virus growth

Bovine coronavirus was grown in mammalian cell culture. For antigen production, cell culture supernatant was harvested and clarified by low speed centrifugation. The virus was then inactivated by treatment with 0.0015 M binary ethyleneimine for 24 h at 37°C. Finally, virus was concentrated up to 50-fold using a hollow fibre filtration system with a 100,000 molecular weight cut-off.

### 2.2. Antigen quantitation

Bovine coronavirus antigen levels were determined by quantitative ELISA. Immulon 4 removable strips (Dynex Technologies) were coated with an optimal concentration (previously determined by titration) of a sheep polyclonal anti-bovine coronavirus antibody diluted in 0.05 M carbonate–bicarbonate coating buffer of pH 9.6. After incubation at 37°C for 1 h, the wells were blocked using PBS containing 0.1% bovine serum albumin and 1% Tween 20 for 10 min at ambient temperature. 100 µl of PBS containing 0.1% Tween 20 was added to each well followed by 50 µl of sample, standard or control. After incubation for 2 h at 37°C on a shaker incubator the wells were washed three times using PBS containing 1% Tween 20. 200 µl of optimally diluted mouse monoclonal antibody specific for the virus haemagglutinin was then added to each well, followed by incubation for 1 h at 37°C with shaking. After washing, 200 µl of an optimal concentration of a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Nordic Immunologicals) was added to each well and incubated with shaking for a further 1 h at 37°C. After washing, bound conjugate was visualised using *o*-phenylenediamine dihydrochloride (Sigma) as the chromagen. After stopping the reaction using 2.5 M sulphuric acid, the optical density (492 nm) was proportional to the amount of bovine coronavirus antigen in the sample. Full quantitation up to a range of 100 antigen units per ml was achieved by including a set of six standards in each assay.

### 2.3. Vaccine preparation

The vaccine comprised of rotavirus and *E coli* K99 antigens from Rotavec K99<sup>™</sup> (Schering Plough Animal Health) plus inactivated bovine coronavirus antigen and was adjuvanted using a mineral oil based adjuvant. The aqueous and oil phases were prepared separately and combined prior to emulsification using a Silverson homogeniser.

### 2.4. Animals and immunisation protocol

#### 2.4.1. Dose response study

Twelve maiden heifers of mixed breeds that had not previously been treated with vaccines containing coronavirus were housed indoors, bedded with straw, although access was given to a grass paddock for exercise. The animals were fed hay, silage and a standard cattle ration (Quinns of Baltinglass), water was available ad libitum. The health of all the animals was monitored by daily observation throughout the study.

All the animals were bled 28 days prior to vaccination, serum prepared and the coronavirus antibody titre determined. The animals were ranked by descending antibody titre, and allocated into four groups of three animals each based on random assignment of one animal from each third of the ranking. Animals in groups 1 to 3 were immunised with 2 ml of the appropriate vaccine. Group 1, vaccine A containing 7.6 antigen units of coronavirus per dose; Group 2, vaccine B containing 38 antigen units of coronavirus per dose and Group 3, vaccine C containing 190 antigen units of coronavirus per dose, animals in group 4 were not immunised.

Vaccinated animals received a single 2 ml injection intramuscularly into the neck. All animals were bled before vaccination, 14, 28 and 84 days post vaccination, heifers in groups 2 and 3 were also bled at 112, 140 and 168 days post vaccination. Blood samples were stored for 12 h to allow clotting to occur, the serum separated by centrifugation, and stored at –20°C prior to testing for bovine coronavirus (BCoV) antibodies by virus neutralisation and haemagglutination inhibition.

#### 2.4.2. Cow/calf studies

These were carried out on four farms, two (studies A and B) were beef suckler herds and two (studies C and D) were dairy herds. A double blind trial design was used. Thirty cows on each farm were paired according to their expected calving dates and then randomly allocated to either vaccinate or placebo treatment groups (15 cows per group). Animals were included in the study on the basis that their expected calving dates fell between 2 and 12 weeks later than the day of vaccination. Animals were excluded only if unhealthy or where they were known to have been previously vaccinated against coronavirus. All animals received a single injection of 2 ml intramuscularly in the neck. Animals in the vaccinate group were immunised with a preparation containing 150 antigen units of coronavirus per dose, whilst animals in the control group were vaccinated with a saline/oil emulsion placebo containing none of the vaccine antigens.

Rectal temperatures were taken from all animals either 2 days before vaccination (studies A and B) or

immediately before vaccination (studies C and D), approximately 1 h after vaccination and 2 days after vaccination. On all sites, injection sites were examined for local reactions 1 day, 2 days and 14 days after vaccination.

Calves from the beef suckler herds (studies A and B) were allowed unrestricted access to their dams for suckling. Calves from the dairy herds (studies C and D) were fed twice a day by hand with approximately 1.5 l of colostrum or milk from their own mothers for at least the first 7 days of life.

Blood samples were collected from all cows at intervals from pre-vaccination to post-calving as indicated in the results section. In studies A and B, milk samples from the dam and blood samples from the calf were collected at 7 day intervals from the day of calving (post-suckling) to 28 days post-calving, whilst in studies C and D samples were only taken on the day of calving (post-suckling) and three days post-calving.

Blood samples were processed as described above. Colostrum or milk samples were stored at  $-20^{\circ}\text{C}$  prior to testing for the presence of BCoV neutralising antibody.

### 2.5. Sample preparation

Prior to testing, all sera were thawed, heat-treated at  $56^{\circ}\text{C}$  for 30 min, sub-aliquoted and stored frozen at  $-20^{\circ}\text{C}$  until required.

Colostrum and milk samples were thawed, centrifuged at  $2000 \times g$  for 10 min and the heavy liquid fraction removed aseptically by a pipette to a clean sterile container. Sterile Rennet solution was then added to a concentration of 10% v/v and incubated for 1 h at  $37^{\circ}\text{C}$ . The mixture was then centrifuged at  $2000 \times g$  for 10 min and the upper liquid fraction (the whey) aseptically removed by pipette and dispensed into sub-aliquots of at least 0.5 ml before storage at  $-20^{\circ}\text{C}$ .

### 2.6. Antibody responses

Bovine coronavirus neutralising antibody responses were quantified using a modification of a standard plaque reduction assay [14]. Viral plaques were visualised by haemadsorption using 1% (w/v) rat red blood cells and the virus neutralising (VN) titre defined as the 50% end point following challenge with 40 plaque forming units of BCoV.

Bovine coronavirus haemagglutination inhibition (HAI) antibodies were quantified as follows. Test sera were first pre-treated to remove non-specific agglutinins and inhibitors by incubation with an equal volume of 2% (w/v) rat red blood cells overnight at  $2-8^{\circ}\text{C}$  followed by treatment with 0.25% (w/v) potassium periodate for 30 min at room temperature. Standard HAI methodology was used, doubling dilutions (50  $\mu\text{l}$

sample + 50  $\mu\text{l}$  PBS containing 0.5% bovine serum albumin) of test samples were carried out in 'V' bottomed microwell plates. An equal volume of BCoV antigen containing four haemagglutinating units was then added and incubated for 1 h at room temperature. 50  $\mu\text{l}$  of 0.5% (w/v) rat red blood cells was then added and the test plates incubated overnight at  $2-8^{\circ}\text{C}$ . The haemagglutination antibody titre was defined as the reciprocal of the highest dilution giving 100% inhibition of agglutination, allowing for the pre-treatment dilution.

## 3. Results

### 3.1. Dose response study

All animals possessed some pre-existing antibody to bovine coronavirus as measured by both HAI (Range  $\log_2 4-6$ ) and VN (Range  $\log_{10} 2.7-4.0$ ). No defined increase in antibody titre could be detected by either HAI or VN in animals immunised with the lowest dose of BCoV (7.6 antigen units). However, animals receiving  $\geq 38$  antigen units of BCoV antigen showed an increase in mean HAI antibody titre by day 14. Peak HAI titres of  $\log_2 7.7$  and  $\log_2 8.5$  were observed either 84 days post-vaccination with 38 antigen units per dose, or 28 days post-vaccination with 190 antigen units per dose, respectively. These animals were maintained for 168 days and showed a steady drop in titre of approximately  $\log_2 0.5$  per month from day 84 (Fig. 1a). The VN antibody response was similar but peaked earlier at day 14 with titres of approximately  $\log_{10} 4.4$ . However, while VN antibody levels declined between days 28 and 84 in cattle receiving 38 antigen units (Fig. 1b), those obtained from animals receiving the higher dose of 190 units did not decline until after day 84. Only the group immunised with 190 antigen units of BCoV still possessed a VN titre  $\geq 1 \log_{10}$  above the pre-immunisation titre at 84 days post-vaccination (Fig. 1b). The sentinel group showed no increase in antibodies to BCoV by either HAI or VN during the study.

As a result of this study, a BCoV antigen load of 150 antigen units per dose was selected for further study on the basis that it was likely to produce an optimal antibody response in terms of antibody titre and duration. Vaccines for the cow/calf studies were therefore formulated at this level.

### 3.2. Cow/calf studies

The vaccine was well tolerated and no significant local or systemic side effects were observed.

The antibody results from these studies are shown in Fig. 2. Since virus neutralisation is the key parameter

involved in protection by lactogenic immunity, antibody titres were only determined by VN. With the exception of study B, the serum VN antibody levels of the placebo groups did not change significantly during the course of the experiment. In study B however, 33% (5/15) of placebo treated animals showed an increase in serum VN antibody levels of  $\log_{10}0.5$ –1.0 during the experiment suggesting that ‘wild type’ BCoV was circulating within the herd. After immunisation, serum VN antibody levels in the vaccinated groups showed a significant ( $p < 0.05$ ) increase in serum VN antibody levels compared with those observed in the placebo control groups. Mean serum VN antibody levels were increased by at least  $\log_{10}0.5$  by 14 days post-vaccination and these levels were typically maintained until calving. Associated with this increase in serum VN antibody levels, first day colostrum VN antibody was also significantly higher ( $\log_{10}0.5$ –0.8) in the test vaccine groups compared with

the placebo control groups. Similar differences were maintained in milk antibody throughout the period of study (up to day 4 on the dairy farms and up to day 28 on the beef suckler farms). The smallest increase occurred in study B where sero-conversion amongst the placebo treated animals was observed (Fig. 2b). Serum taken from calves allowed to suckle vaccinated dams showed that levels of circulating VN antibody were up to ten-fold higher than those obtained from calves suckling placebo-vaccinated cows.

The effect of the interval between vaccination and calving on the increase in milk VN antibody titre can be seen in Table 1 where data from studies A and B have been combined into five sets, each set comprising 12 cows (three vaccinate and three placebo cows from each study) with mean vaccination/calving intervals of 19, 30, 46, 69 and 85 days, respectively. For placebo treated animals, irrespective of the vaccination/calving interval, day 0 colostrum possessed VN titres of ap-

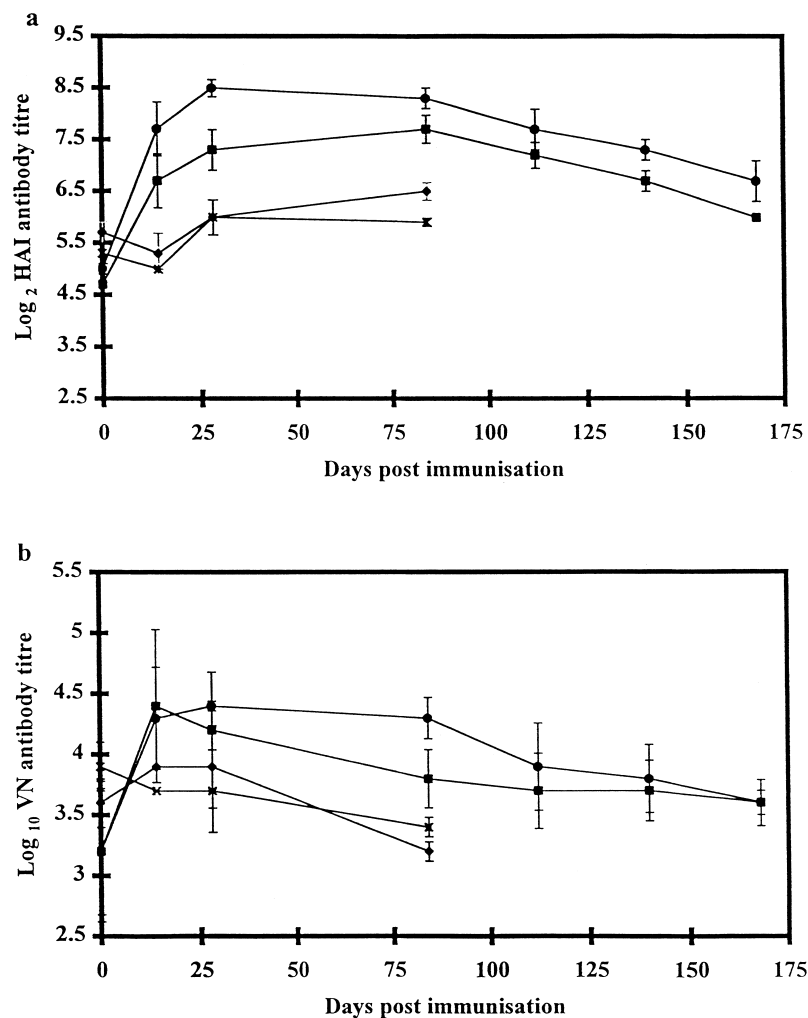


Fig. 1. BCoV antibody response following immunisation with combined *E. coli*, rotavirus and coronavirus vaccines containing different concentrations of BCoV antigen (● 190 units per dose, ■ 38 units per dose, ◆ 7.6 units per dose, × unvaccinated controls). Graphs show the specific serum antibody responses as measured by HAI (a) and VN (b). Error bars denote 95% confidence limits.

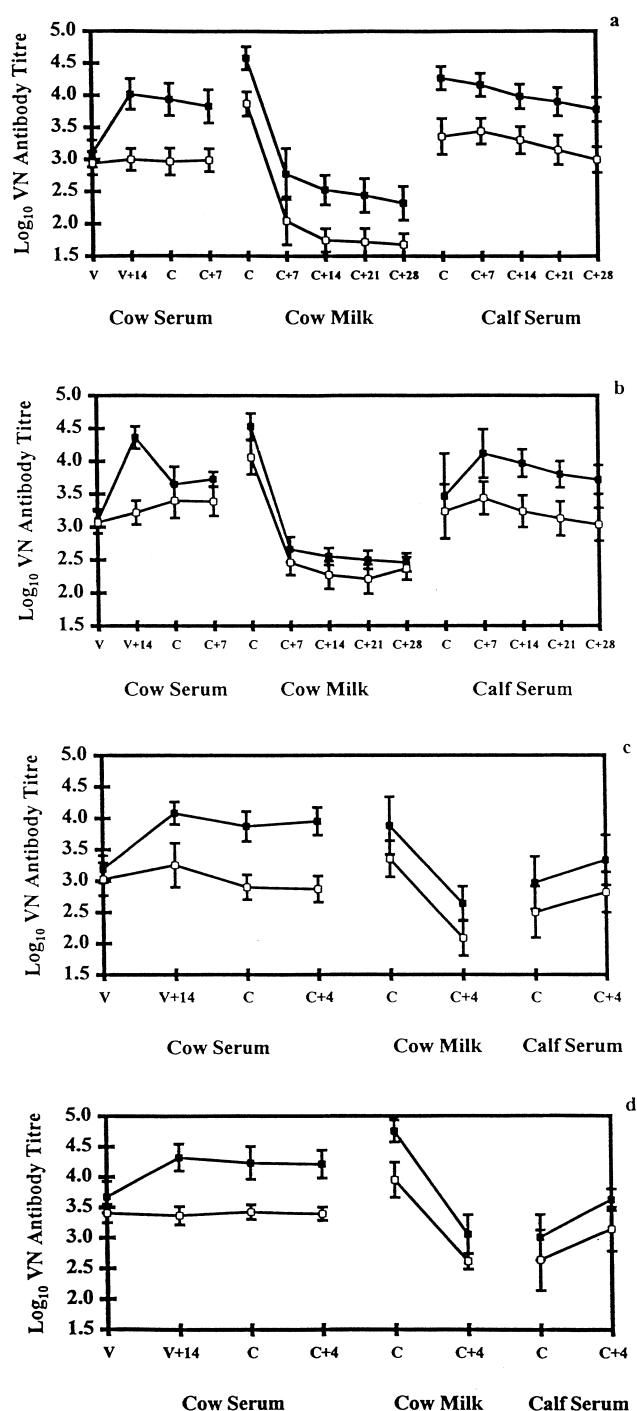


Fig. 2. Mean BCoV VN antibody results in sera and milk from vaccinated (■) and placebo treated (□) cows and sera from their respective calves. Studies were carried out on four farms, two beef suckler herds (a and b) and two dairy herds (c and d) and samples were obtained at vaccination (V), calving (C) or at the number of days indicated after the event. Error bars denote 95% confidence limits.

proximately  $\log_{10}4.0$ , falling rapidly to levels of approximately  $\log_{10}2.0$  (background) in day 7 milk. In contrast, for the vaccinated cattle, day 0 colostrum possessed VN titres of approximately  $\log_{10}4.5$ , with a much slower reduction in antibody level, only approaching background levels in 28-day milk. The duration of the milk antibody response seen in the vaccinated animals where the vaccination/calving interval was 19 days was less marked than where this interval was longer. This reflects a reduced response to vaccination seen in approximately 50% of the vaccinates in the 19-day set.

#### 4. Discussion

BCoV antigen input into the formulated vaccine was measured by ELISA, since measurements based on infectivity do not quantitate total antigenic mass, only viable virus particles and therefore could not be used post-inactivation. The ELISA procedure used was primarily targeted towards the viral haemagglutinin which has been shown to contain a key virus neutralising epitope [15]. Antigen levels could therefore be tracked throughout the formulation processes.

The results of the dose response study indicate that in the presence of pre-existing antibody, relatively large amounts of immunogen ( $>38$  antigen units) are needed to stimulate a significant increase in BCoV serum antibody titre. There appear to be some minor differences in the pattern of the antibody responses determined by either HAI or VN in that the HAI responses peak later than the VN responses. This may reflect differences in antibody affinity or sub-class requirements between the two methods of antibody measurement. In terms of neutralising antibody, the major effect of increasing antigen content appears to be in the duration rather than the magnitude of the response. With a vaccine dose containing 190 antigen units of antigen the response was rapid, reaching a plateau approximately 14 days post-vaccination which was maintained until at least 84 days post-vaccination. With a dose containing 38 antigen units of antigen, the virus neutralisation response was equally rapid and of similar magnitude, although in contrast declining rapidly to 84 days post-vaccination. Selection of the correct antigen load therefore appears critical in ensuring an optimal window in terms of the interval between vaccination and calving. An additional advantage in using a higher antigen dose was also demonstrated in cow / calf study B where elevated antibody responses were still observed even in the face of circulating infection. This suggests that vaccination could still play a significant role in enhancing overall herd immunity even in an active disease situation.

The main source of antibody in bovine mammary secretions is serum IgG<sub>1</sub>. This is selectively transferred from serum throughout lactation, albeit at a reduced level in milk compared to colostrum [16]. The initial serology data therefore suggested that with a BCoV antigen load of approximately 190 antigen units, successful vaccination could take place at any time between 2 and 12 weeks pre-calving. This hypothesis was supported by the observations on the magnitude of the antibody titres found in the colostrum and milk of cows vaccinated at different times pre-calving with a vaccine containing 150 antigen units of BCoV. Significant responses in colostrum and milk were observed at both ends of the vaccination window, although not all cows vaccinated approximately 14 days pre-calving appeared to show an optimal response.

Individually, there was a large variation in the levels of specific BCoV antibody present in both vaccinate and placebo control groups throughout the study. This variability was particularly noticeable in colostrum and milk samples, and presumably arose from the differential levels of pre-existing BCoV antibodies observed in the animals. Although this would be expected to adversely affect the statistical testing of the results, significant differences ( $p > 0.05$ ) between test and control groups were found on the majority of days tested. This contrasts strongly with previously reported studies wherein increases in BCoV antibody titre in serum or milk following vaccination were either minimal or non-existent [9–13]. The reasons for the improved responses observed with the test vaccine are related both to the concentration of BCoV antigen present and the use of an oil adjuvant, which have generally been reported to be highly effective in the

enhancement of rotavirus antibody titres in mammary secretions [8].

The magnitude of the antibody response observed following a single vaccination may be the result of the use of pre-primed (as a result of previous infection) cattle. Bovine coronavirus is ubiquitous and in the field situation it is extremely difficult to find a seronegative individual. This observation is supported by the data presented in this study where a total of 132 heifers and cows, with no previous BCoV vaccination history, were tested prior to treatment and all were found to have substantial levels of pre-existing antibody.

A major decline in specific antibody levels present in mammary secretions was observed in the first few days after calving. This is consistent with previously reported research [7,17], which showed that the switch from colostrum to milk was associated with a decrease in immunoglobulin concentration. Enhanced antibody levels are maintained for 21 to 28 days post-calving, although as BCoV specific antibody levels decrease calves will become more susceptible to sub-clinical infection. Such infections will stimulate active immunity and hence confer long term protection upon the calf.

In the beef suckler situation, where the calf is allowed to suckle naturally from its dam, it is relatively easy to ensure the continual presence of protective antibody in the gut lumen. In the dairy situation, typically the calf is allowed access to colostrum but is prevented from suckling milk and thus fails to ensure the presence of protective antibody in the gut lumen beyond the first few days after birth. A modified feeding regimen is therefore required whereby colostrum

Table 1

The effect of the interval between vaccination and calving on the magnitude of the BCoV VN antibody response in mammary secretions

Mean log<sub>10</sub> BCoV VN antibody titre in colostrum/milk following vaccination at the specified time before calving

Sample time	Treatment group	19 Days	30 Days	46 Days	69 Days	85 Days	Mean of all cows
Calving	Vaccinate	4.31	4.47	4.50	4.70	4.70	4.55 <sup>a</sup>
	Placebo	3.72	3.96	3.93	4.02	4.12	3.95
C + 7	Vaccinate	2.63	2.44	2.66	2.66	3.19	2.71 <sup>a</sup>
	Placebo	2.19	2.03	2.16	2.36	2.50	2.25
C + 14	Vaccinate	2.16	2.70	2.40	2.57	2.78	2.53 <sup>a</sup>
	Placebo	2.01	1.86	1.85	2.15	2.12	2.00
C + 21	Vaccinate	2.19	2.61	2.29	2.46	2.78	2.47 <sup>a</sup>
	Placebo	1.82	1.86	1.99	2.01	2.06	1.95
C + 28	Vaccinate	2.13	2.42	2.11	2.49	2.71	2.39 <sup>a</sup>
	Placebo	1.89	2.12	2.11	1.96	1.93	2.01

<sup>a</sup> Indicates significant difference from placebo treated group at  $p < 0.001$  (Student's *t*-test).

feeding is continued as part of the calves' diet for at least the first 2 weeks of life.

The levels of specific antibody present in the colostrum samples from the dairy herds (studies C and D) showed a marked drop in titre over 3 days, this was associated with the switch from colostrum to milk but may be further exacerbated by a dilution effect related to the larger volumes of milk produced by dairy cattle compared with beef cattle. These antibody levels were however significantly higher than those in the placebo treated group and equivalent to those seen in milk from beef cattle at 7 days post-calving. Continued feeding of colostrum pooled from collections obtained during the first 3 days post-calving should therefore provide an enhanced level of protection to the calf over this time period.

It is accepted that by analogy with rotavirus infections in the calf [18] and transmissible gastro-enteritis virus infections in the piglet [19], lactogenic immunity should protect against BCoV infection in the calf [20], and this has been confirmed experimentally (Crouch C.F., unpublished observation). There are however significant problems in assessing the efficacy of vaccines where protection is based on boosting lactogenic immunity. This is a result of the number of variables that interact to effect the apparent level of protection obtained. Such variables include the management system for the target animal, the titre of specific antibody achieved in both colostrum and milk, the duration of the enhanced antibody response, the volume and timing of antibody ingested and the nature (amount and pathogenicity) of the challenge. Of these, the key parameters able to be influenced directly by the design of the vaccine are the titre and duration of specific antibody achieved in colostrum and milk.

This report indicates that a single dose of coronavirus vaccine administered to the pregnant heifer 2 to 12 weeks before calving is capable of significantly increasing the titre and duration of specific antibody present in colostrum and milk. Work is currently underway to confirm the minimum level of specific antibody required to protect the calf from challenge and thus establish the duration of immunity.

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