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Serological, colostral and milk responses of cows vaccinated with a single dose of a combined vaccine against rotavirus, coronavirus and *Escherichia coli* F5 (K99)

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Twenty-five Ayrshire/Friesian cows were vaccinated once with a new combined vaccine against rotavirus, coronavirus and *Escherichia coli* F5 (K99) or given a saline placebo 31 days before the first expected calving date. Blood samples were taken from the cows at intervals from vaccination until seven days after calving and from their calves up to 28 days after birth, and colostrum and milk samples were collected from the cows at intervals for 28 days after calving. There was a significant increase in the mean specific antibody titre against all three antigens in the serum of the vaccinated animals (even in the presence of pre-existing antibody) which was accompanied by increased levels of protective antibodies to rotavirus, coronavirus and *E coli* F5 (K99) in their colostrum and milk for at least 28 days.

NEONATAL calf diarrhoea is a complex disease associated with a number of infectious agents occurring either singly or in combination. Rotavirus appears to be the commonest cause followed by Cryptosporidium species, coronavirus and enterotoxigenic Escherichia coli (ETEC) (Snodgrass and Browning 1993). Rotavirus and coronavirus are both ubiquitous in the environment and in addition to causing clinical disease in neonates, can be associated with subclinical infection in adult animals (Crouch and Acres 1984), which may therefore act as reservoirs for reinfection. Passive immunity against enteric viral infection is mediated by the continual presence of a protective level of specific anti-viral antibody in the gut lumen (Crouch 1985, Snodgrass and Browning 1993). Calves can be protected against ETEC by the stimulation of high levels of colostral antibody against the F5 or K99 pilus antigen which has been shown to play a key role in the pathogenicity of ETEC (Acres and others 1979, 1982, Krogh 1983).

This paper describes the increases in the level and persistence of antibodies in colostrum and milk against rotavirus, coronavirus and ETEC F5 (K99) antigens after cows had been vaccinated with a single dose of a new combined vaccine.

MATERIALS AND METHODS

Vaccine

The vaccine was Rotavec Corona (Schering-Plough Animal Health), a commercially available inactivated vaccine for use as an aid in the protection of calves against diarrhoea by the transfer of passive immunity from the dam. It consists of a combination of the antigens of inactivated bovine rotavirus (serotype G6 P5), inactivated bovine coronavirus (originally isolated from a calf with diarrhoea) and purified cell-free *E coli* F5 (K99) (adsorbed on to aluminium hydroxide gel), and is formulated as an emulsion in a light mineral oil.

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Study design

Twenty-five Ayrshire/Friesian cows which had not been vaccinated against rotavirus, coronavirus or *E coli* were allocated to two treatment groups balanced according to their expected calving dates. Fifteen animals were vaccinated and 10 were used as unvaccinated controls. A single dose (2 ml) of the vaccine or saline control was administered intramuscularly into the left shoulder/neck region of each animal, 31 days before the first expected calving date, and the animals were examined for signs of local or systemic reactions after approximately one hour, and after three, seven and 14 days. The cows calved naturally and the calves were allowed to suckle for up to 24 hours; they were then fed a commercial milk replacer as recommended by the manufacturer.

Sample collection and processing

Colostrum and milk samples (20 ml) were collected when possible from the cows within six hours of calving before the calf had suckled, and seven, 14, 21 and 28 days after calving. Each sample was centrifuged at 1000 g for 10 minutes and the liquid fraction was treated with rennet solution (Langdales) to a final concentration of 10 per cent. This mixture was then incubated for an hour at 37°C after which it was again centrifuged at 1000 g for 10 minutes. The upper liquid fraction (whey) was stored in two plastic Bijoux bottles at -20° C.

Blood samples were taken from the cows when they were vaccinated, 14 days before the first expected calving date, at calving and seven days after calving, and from the calves on the day of birth and seven, 14, 21 and 28 days after birth. Serum samples were collected aseptically from the clotted blood, transferred into duplicate tubes and stored at -20° C.

Antibody determinations

Specific viral antibody titres in serum and whey were determined by virus neutralisation with bovine rotavirus serotypes G6 and G10 and bovine coronavirus, using the standard method described by Snodgrass and others (1982). The neutralisation tests were read by specific immunofluorescence with the end-point defined as the reciprocal of the highest sample dilution giving a 60 per cent reduction in the number of fluorescing cells. Control sera were included in each assay to ensure the equivalence of the results of different assays.

Specific antibody to *E coli* F5 (K99) was determined by means of a competitive ELISA. Purified F5 antigen (Karkhanis and Bhogal 1986) was coated indirectly on to the solid phase by using a polyclonal anti-F5 antibody. The degree of binding of an anti-F5 monoclonal antibody (mAb) in the presence and absence of the test sample was determined by using an anti-mouse horseradish peroxidase conjugate and the reaction was developed with tetramethylbenzidine K blue as substrate. The reaction was stopped after 10 minutes with 0·025M sulphuric acid and the optical density (OD) at 450 nm was determined for each well. The ability of the unknown Mean percentage inhibition of binding

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TABLE 1: Statistical significance of differences between specific mean colostrum or milk antibody titres from vaccinated and placebo treated cows				
Days after calving		inated and con colostrum and	trol groups for milk antibodic Rotavirus G6	specific 15
0	<0-001	0-002	0-002	NS
7	0-01	0-012	0.003	0-003
14	0-03	0-028	NS	NS
21	0-02	0.012	0-037	0-006
28	0-02	<0-001	0-008	0-006

NS Not significant

sample to inhibit the binding of the mAb was expressed as follows:

per cent inhibition = 100-[(sample binding/total binding) × 100]

where the total binding is the mean OD of the reaction mixture in the absence of any sample, and the sample binding is the mean OD of the reaction mixture in the presence of a sample.

Control sera (positive and negative) were included in each assay to validate the test.

Statistical analysis

The different methods used to determine the antibody titres to rotavirus and coronavirus as opposed to $E \, coli \, F5 \, (K99)$ gave rise to data sets with different properties. The virus antibody data sets were analysed by using a generalised linear model (proportional odds model – Minitab version 11.11). The $E \, coli \, F5 \,$ data set was analysed by analysis of covariance (Genstat 5 release 3.22). A P value of <0.05 was taken to indicate statistical significance.

RESULTS

E coli F5 (K99) antibody response

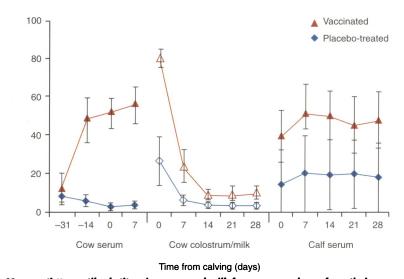
After they were vaccinated, the mean specific antibody levels in the cows' sera had increased significantly compared with the controls (48.4 per cent v 5.8 per cent; P<0.001) by at least 14 days before calving, and these levels were maintained throughout the study (Fig 1). Assuming that an inhibition of binding in the ELISA of more than 20 per cent can be regarded as significant, two animals in the vaccinated group had significant levels of antibody before they were vaccinated; one had an antibody value of 25.1 per cent which increased to approximately 73 per cent inhibition by 14 days before calving, and the other had an antibody value of 68.4 per cent inhibition which increased to approximately 85 per cent inhibition by 14 days before calving. In both cases these levels were maintained throughout the study.

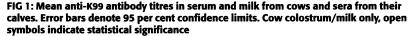
There were significantly higher levels of specific antibody in the colostrum and milk of the vaccinated animals than in the controls throughout the 28 days after calving (Table 1). The difference was larger in the colostrum taken on the day of calving than in the later milk samples (Fig 1).

The calves from the vaccinated dams also had higher serum antibody levels than the calves from the control dams (Fig 1), although in three animals the difference did not become apparent until after seven days.

Coronavirus antibody response

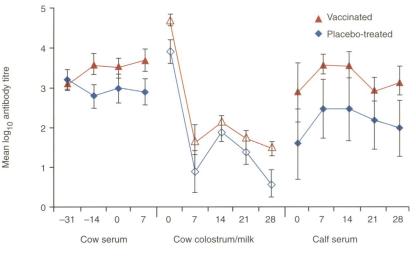
All the cows had titres of serum antibody ranging from \log_{10} 2.8 to \log_{10} 4.3 before they were vaccinated. After vaccination, the mean specific antibody levels increased significantly com-





pared with those in the control animals (14 days before calving, P=0.002; at calving, P=0.005; seven days after calving, P=0.003). The increase of at least \log_{10} 0.5 was maintained throughout the study (Fig 2). None of the animals in the control group showed a significant (four-fold) increase in antibody titre during the study. In contrast, 13 of the 15 cows in the vaccinated group showed significant increases in antibody titres, and one of the remaining two had a prevaccination antibody titre of $\log_{10} 3.1$ and the other a titre of $\log_{10} 3.7$, the highest titre in this group.

There were also significantly higher mean levels of antibody in the colostrum and milk of the vaccinated cows than of the controls (Table 1). Similarly higher levels were maintained for at least 28 days after calving (Fig 2). Animals with high levels of circulating antibody tended to have higher levels of colostral (and milk) antibodies. The calves from vaccinated dams also had higher serum antibody levels than the calves from the control dams (day 0, P=0.020; day 7, P=0.005; day 14, P=0.011; day 21, P=0.041; day 28, P=0.012; Fig 2). In four calves the difference did not become apparent until after



Time from calving (days)

FIG 2: Mean coronavirus neutralising antibody titres in serum and milk from cows and sera from their calves. Error bars denote 95 per cent confidence limits. Cow colostrum/milk only, open symbols indicate statistical significance

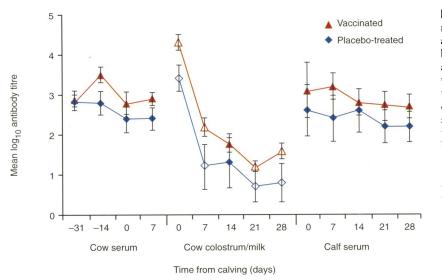


FIG 3: Mean rotavirus (G6) neutralising antibody titres in serum and milk from cows and sera from their calves. Error bars denote 95 per cent confidence limits. Cow colostrum/milk only, open symbols indicate statistical significance

seven days, and three of these calves were the same as those which failed to show a difference in antibody levels to *E coli* F5 (K99) until after seven days.

Rotavirus antibody response

Before they were vaccinated all the animals had serum antibody titres against the homologous (G6) rotavirus serotype ranging from $\log_{10} 1.6$ to $\log_{10} 3.1$. After vaccination, the mean rotavirus G6 neutralising antibody levels in the cows' sera had increased significantly by 14 days before calving, compared with the control group (P=0.002), and this increase was maintained throughout the study (Fig 3). The responses of individual animals varied, those with previously low antibody levels showing increases in neutralising antibody titres of up to 128-fold, whereas some animals with previously high antibody levels ($\log_{10} 3.1$) failed to show a significant (>four-fold) response.

There were higher mean levels of rotavirus G6 neutralising antibodies in the colostrum and milk from the vaccinated cows than from the control cows for at least 28 days after calving (Fig 3). Furthermore, the levels of both G6 and G10 serotypes were significantly higher at selected times after calving (Table 1). Cows with higher levels of circulating antibody tended to have higher levels of colostral and milk antibodies.

The calves from the vaccinated dams had higher rotavirus G_6 neutralising serum antibody levels than the calves from the control dams although the difference was statistically significant only seven days after birth (P=0.014) (Fig 3).

DISCUSSION

Both rotavirus and coronavirus invade and damage the mature cells of the villus epithelium; as a result the presence of specific antibody in the lumen of a calf's intestine can exert a protective effect by virus neutralisation. With ETEC, the bacteria are localised by fimbrial adhesins, predominantly F5 (K99), on the mucosal surface where they secrete enterotoxins (Tzipori 1985, Snodgrass and Browning 1993). The presence of specific anti-F5 antibodies in the lumen of a calf's intestine exerts a protective effect by preventing the initial attachment of the bacterium. In order to ensure that none of these agents induce clinical disease it is important that colostrum or milk from the dam containing adequate

levels of specific protective antibody is ingested. The vaccination of these cows with a single dose of the commercially available inactivated vaccine containing a combination of bovine rotavirus, bovine coronavirus and purified E coli K99 antigens increased the levels of the specific antibodies to these important pathogens in both colostrum and milk. That these antibodies were available to protect the calf was demonstrated by the detection of antibody in the calves serum, which although not critical to protection indicated that the calves had suckled from their mothers soon after birth. These results agree with those of Acres and others (1979), Nagy (1980), Snodgrass and others (1980, 1982), Hess and others (1982), Castrucci and others (1984), Saif and others (1984) and Snodgrass (1986) who showed that the vaccination of cows with either E coli F5 (K99) antigen or rotavirus antigen alone or in combination induced levels of antibody in the milk which could provide significant protection against challenge. However, previous studies (Dauvergne and others 1983, Stepanek and others 1987, Wieda and others 1987, Mostl and Burkl 1988, Kohara and others 1997) have described only minimal increases or no increases in the levels of coronavirus antibody in either serum or milk after the vaccination of cows with preparations containing inactivated bovine coronavirus antigen, whereas the vaccine used in this study produced a significantly enhanced antibody response in the milk. The reasons for this difference are unclear but are most likely to be associated with the levels of coronavirus antigen incorporated into this new vaccine and the use of a highly effective adjuvant (Crouch 1985).

It is recognised that, as a result of the number of variables that interact to affect the apparent level of protection obtained, there are significant problems in assessing the efficacy of vaccines in the field, where protection is based on enhancing the antibody response in the milk. Such variables include the management of the vaccinated animal, the titres of specific antibody achieved in colostrum and milk, the duration of the enhanced antibody response, the volume of antibody ingested and its timing, and the size and pathogenicity of the challenge. The variables most readily influenced by the use of a new vaccine are the titre and duration of the specific antibody levels achieved in colostrum and milk. Comparisons between different studies are further compounded by technical differences in the methods used to quantify specific antibody levels. For example, Nagy (1980) used a microtitre plate bacterial agglutination test, Acres and others (1979) used a radioimmune assay and Snodgrass and others (1982) used a combination of an indirect ELISA and passive haemagglutination to quantify K99 antibody. It is therefore not possible to make a direct comparison between the antibody levels observed in this study and those previously shown to be protective. Nevertheless, the levels of antibody detected in colostrum and milk against each antigen by using standard methods increased significantly after the vaccination, and this is a prerequisite for the provision of protective levels of immunity to the suckling calf.

The increase in the level of *E coli* F5 (K99) antibody after vaccination was more pronounced in colostrum than in milk. However, in contrast with the disease caused by rotavirus or coronavirus, which typically peak five to seven days after birth, ETEC infections tend to cause a severe, watery diarrhoea at one to two days of age (Moon and others 1978). This early susceptibility to ETEC infection is due in part to the relatively low incidence of disease in adult cows, resulting in negligible specific antibody titres in normal colostrum and leaving the newborn calf fully susceptible. In addition, age-related resistance to F5-mediated ETEC adhesion to the intestine has been demonstrated in vitro in both calves and piglets (Runnels and others 1980). With

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such early susceptibility to disease, colostral antibodies play a more significant role than milk antibodies, in controlling ETEC infections. However, with rotavirus or coronavirus diseases it is equally important to ensure that the protective levels of antibody last longer. In the case of suckling calves this can be achieved by maintaining a high level of specific antibody in the milk for as long as possible. Higher levels (>four-fold) of specific rotavirus and coronavirus antibodies were found in the milk of the vaccinated cows than in the control cows for at least 28 days after calving, suggesting that the required levels of virus-neutralising antibodies would be available to calves for a significant time after birth. The rapid decline in specific antibody levels observed between those in the colostrum on the day of birth and those in the milk seven days later is a result of the more than 50-fold lower rate of selective transfer of IgG₁ from serum to milk compared with its transfer to colostrum (Butler 1973, Newby and others 1982).

The levels of rotavirus G10-specific antibody in the colostrum and milk of the vaccinated cows also tended to be higher, probably as a result of the production of heterotypic antibodies by animals that had been infected by a broad spectrum of rotavirus serotypes (Snodgrass and others 1984, Brussow and others 1988, Green and others 1990).

The blood samples taken before the cows were vaccinated showed that some of them were already seropositive for coronavirus, rotavirus and anti- $E \, coli \, F5 \, (K99)$ antibody. Sufficient antigen must therefore be present in a vaccine to overcome the effects of these antibodies and induce an immune response. Only the cows already having the highest antibody levels (presumptively recently exposed) failed to respond serologically to the single dose of vaccine, and they produced a response of equivalent titre and duration in their colostrum and milk to those produced by the cows which did respond serologically.

The results of these studies show that a single inoculation of pregnant cows with the combined rotavirus, coronavirus, E coli F5 (K99) vaccine significantly improved the level of passive immunity in their calves for at least 28 days. The data also show that overall herd immunity could be improved by bringing the titres of antibodies in colostrum and milk up to a similar level, a procedure which should help to control these three significant causes of neonatal diarrhoea in cattle, when it is used as part of a good farm management programme.

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