NEW MICROBIOLOGICA, **32**, 109-113, 2009

A candidate modified-live bovine coronavirus vaccine: safety and immunogenicity evaluation

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SUMMARY

A modified-live vaccine against the respiratory form of bovine coronavirus (BCoV) infection was developed by progressive attenuation of a respiratory strain (438/06-TN). The vaccine was found to be safe as four colostrum-deprived newborn calves remained healthy after oronasal administration of ten doses of the vaccine. The immunogenicity of the vaccine was assessed by intramuscular injection of one vaccine dose to 30 BCoV-antibody negative 2-3-month-old calves. At 30 days post-vaccination, all vaccinated calves displayed high antibody titres against BCoV. Sequence analysis of the S gene of wild-type and cell-adapted 438/06-TN strain detected 10 nucleotide changes, 9 of which were non-synonymous.

KEY WORDS: Cattle, Respiratory coronavirus, Vaccine

Received May 06, 2008 *Accepted* June 10, 2008

Bovine coronavirus (BCoV), a subgroup 2a coronavirus, is the traditional agent of different forms of enteritis in cattle, including severe diarrhoea in newborn calves (Snodgrass *et al.*, 1986) and winter dysentery in adult cows (Saif *et al.*, 1991). Recently, BCoV infection has also been associated with bovine infectious respiratory disease (BIRD) in calves and adult cattle (Lathrop *et al.*, 2000; Decaro *et al.*, 2008a). Prophylaxis of BCoV enteric disease in newborn calves is usually obtained by means of vaccination of pregnant cows to enhance the level of maternal antibodies that are transferred to their offspring through colostrum, thus exerting a local protective effect against BCoV-induced enteritis (Saif *et al.*, 1983; Kohara *et al.*, 1997). More recently, inactivated vaccines against winter dysentery have been developed for prevention of epidemic diarrhoea in adult cattle (Takamura *et al.*, 2002). To date, vaccines against the BCoV-induced BIRD have not yet been licensed, although the intranasal administration of a modified-live virus (MLV) vaccine prepared with an enteric BCoV was found to reduce the risk of BIRD in vaccinated animals significantly. The objective of the present study was to develop a respiratory BCoV-based vaccine to be employed for prevention of BIRD in cattle herds. The prepared vaccine was evaluated for its safety and immunogenicity in newborn or young calves.

Human rectal tumour (HRT-18) and Madin Darby bovine kidney (MDBK) cells were used for virus isolation and attenuation through cell passages, respectively. Strain 438/06-TN was isolated from the nasal swab of a calf with respiratory disease containing 1.11×10^6 copies of BCoV RNA per µl of template (Decaro *et al.*, 2008a). After two passages on HRT-18 cells, viral attenuation was

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obtained through repeated passaging on MDBK cells. Viral growth was monitored constantly by an immunofluorescence (IF) assay using a BCoV-positive bovine serum and a rabbit anti-bovine IgG conjugated with fluorescein isothiocyanate (Sigma Aldrich srl, Milan, Italy). The 50th passage on MDBK cells (438/06-TN-50) was stored at -70°C in aliquots of 1 or 10 ml and shown to have a viral titre of $10^{8.90}$ $TCID_{50}$ and $8.32x10^{12}$ RNA copies per ml of viral suspension, as calculated by titration on cell cultures and real-time RT-PCR (Decaro *et al.*, 2008d), respectively.

The stock vaccine was tested for sterility from aerobe and anaerobe bacteria, mycoplasmas and mycetes using standardised methods. The presence of contaminant viruses was also ruled out by means of standardised molecular assays for detection of bovine viral diarrhoea virus, bovine herpesviruses types 1 and 4, bovine respiratory syncytial virus, rotaviruses and caliciviruses. A total of 42 animals were used for vaccine evaluation, including six colostrum-deprived 1-day-old newborns and 36 BCoV-antibody negative 2-3-month-old calves. The six newborn calves were housed at the Infectious Diseases Unit of the Animal Hospital of the Faculty of Veterinary Medicine of Bari (Italy), whereas the oldest calves were allocated in a dairy cattle farm in Calabria, Italy. All calves had tested negative for the presence of BCoV RNA in the faeces by real-time RT-PCR (Decaro *et al.*, 2008d) and for BCoV antibodies in the sera by ELISA and haemagglutination inhibition (Decaro *et al.*, 2008b). For the safety test, four colostrum-deprived 1-day-old newborn calves were administered oronasally 10 ml of virus 438/06-TN-50 (corresponding to 10 times the maximum viral titre contained in the 1-ml dose), whereas the remaining two newborn calves were used as controls by receiving by oronasal route 10 ml of saline. All animals were monitored daily for 21 days for the occurrence of fever, diarrhoea, respiratory distress and other signs of BCoV-related illness. Weights of vaccine-treated and control animals were also recorded on a weekly basis, whereas white blood cell (WBC) counts were carried out at days -1 and -3 prior to and 3, 5, 7 and 10 after the virus administration. To confirm the successful infection of the inoculated animals, faecal and nasal swabs were collected daily for BCoV detection by means of real-time RT-PCR (Decaro *et al.*, 2008d), whereas the same animals were bled at day 30 post-inoculation for serology. For the immunogenicity test, 30 BCoV-antibody negative calves, with an age between two and three months, were inoculated intramuscularly in the side of the neck with 1 ml of vaccine. An additional six animals served as unvaccinated controls by intramuscular injection of 1 ml of saline. Serum samples were collected from the animals 30 days post-vaccination.

Sera from animals used for safety and immunogenicity evaluation were subjected to ELISA, haemagglutination inhibition (HI) and virus neutralisation (VN) tests for the detection of BCoV antibodies. The ELISA test was carried out as previously described (Decaro *et al.*, 2007, 2008b), considering a cut-off value of 0.042 (Decaro *et al.*, 2008b). For HI, twofold dilutions (from 1:10 to 1:640) of each serum sample in PBS were made in 96-well V-plates, adding 25 µl of BCoV antigen (corresponding to 8 HA units) to each serum dilution. After an incubation period of 45 min at room temperature, 50 µl of a 0.7% suspension of mouse erythrocytes was added to each well. HI test results were read after 2 h of incubation at room temperature.

The HI antibody titres were expressed as the reciprocals of the highest dilutions of the test sera that inhibited completely the haemagglutinating activity of the virus. For VN, duplicates of serial twofold dilutions of heat-inactivated calf sera (starting from dilution 1:2) were mixed with 100 $TCID_{50}$ of strain 339/06 (Decaro *et al.*, 2008b) in 96-well microtitre plates. After preincubation at room temperature for 90 min, 20,000 MDBK cells were added to each well. The plates were read after 4 days of incubation at 37°C. VN titres were calculated with the Karber method and expressed as the highest serum dilution that was able to neutralise the virus.

The spike (S) protein gene sequences of the BCoC strain used for vaccine preparation was amplified from the original nasal swab (438/06-TN) and after 50 cell passages (438/06-TN-50) using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies, Invitrogen. Milan, Italy), according to the manufacturer's instructions. Four partially overlapping fragments were amplified using primer pairs designed on conserved regions among BCoV-like group 2 coronaviruses (Decaro *et al.*, 2008c). The PCR-amplified products were sequenced by Genome

TABLE 1 - *Detection of BCoV antibodies in animals used for safety and immunogenicity tests.*

Test	*Calf No.*	*Treatment group*	*Route of administration*	*ELISA OD values*	*HI titres*	*VN titres*
S	1	Virus inoculated	O/N	0.164	80	64
S	2	Virus inoculated	O/N	0.149	80	32
S	3	Virus inoculated	O/N	0.099	20	8
S	4	Virus inoculated	O/N	0.110	40	32
S	5	Control	O/N	0.017	<10	<2
S	6	Control	O/N	0.009	<10	<2
I	1	Virus inoculated	IM	0.234	160	128
I	2	Virus inoculated	IM	0.187	40	16
I	3	Virus inoculated	IM	0.177	40	16
I	4	Virus inoculated	IM	0.156	80	16
I	5	Virus inoculated	IM	0.198	80	64
I	6	Virus inoculated	IM	0.214	80	32
I	7	Virus inoculated	IM	0.256	40	32
I	8	Virus inoculated	IM	0.141	80	64
I	9	Virus inoculated	IM	0.169	80	64
I	10	Virus inoculated	IM	0.175	20	16
I	11	Virus inoculated	IM	0.140	40	16
I	12	Virus inoculated	IM	0.165	40	32
I	13	Virus inoculated	IM	0.152	40	16
I	14	Virus inoculated	IM	0.139	160	64
I	15	Virus inoculated	IM	0.171	80	32
I	16	Virus inoculated	IM	0.188	80	64
I	17	Virus inoculated	IM	0.193	80	32
I	18	Virus inoculated	IM	0.204	80	64
I	19	Virus inoculated	IM	0.218	160	128
I	20	Virus inoculated	IM	0.171	40	64
I	21	Virus inoculated	IM	0.126	40	8
I	22	Virus inoculated	IM	0.222	80	128
I	23	Virus inoculated	IM	0.119	20	8
I	24	Virus inoculated	IM	0.143	40	16
I	25	Virus inoculated	IM	0.177	40	32
I	26	Virus inoculated	IM	0.172	80	32
I	27	Virus inoculated	IM	0.196	80	64
I	28	Virus inoculated	IM	0.201	40	64
I	29	Virus inoculated	IM	0.237	80	128
I	30	Virus inoculated	IM	0.180	40	16
I	31	Control	IM	0.021	<10	<2
I	32	Control	IM	0.010	<10	<2
I	33	Control	IM	0.013	<10	<2
I	34	Control	IM	0.025	<10	<2
I	35	Control	IM	0.017	<10	<2
I	36	Control	IM	0.013	<10	<2

S, safety; I, immunogenicity; OD, optical density; HI, haemagglutination inhibition; VN, virusneutralisation; O/N, oronasal; IM, intramuscular. HI and VN antibody titres are expressed as the reciprocals of the highest serum dilutions that completely inhibited viral haemagglutination and growth on cell cultures, respectively.

Express (Meylan, France) and the obtained sequences were assembled and analyzed using the BioEdit software package (Hall, 1999) and the NCBI's (htttp://www.ncbi.nlm.nih.gov) and EMBL's (http://www.ebi.ac.uk) analysis tools.

All colostrum-deprived animals remained healthy throughout the observation period. Appetite was retained and rectal temperatures remained within normal scores, whereas weight gains were similar to those observed in control calves. WBC counts did not displayed remarkable movements from baseline values (data not shown). The inoculated newborn calves shed BCoV RNA with their nasal secretion and faeces. By real-time RT-PCR, BCoV RNA was detected in the nasal swabs for 15 days from days 2 to 16 post-inoculation. In con-

trast, BCoV shedding with the faeces occurred for 9 days, from days 5 to 13 post-inoculation. At 30 days after inoculation, all newborn calves had detectable levels of BCoV antibodies (Table 1). At 30 days post-vaccination, all vaccinated calves displayed high antibody titres against BCoV, as demonstrated by ELISA, HI and VN tests (Table 1). ELISA OD values ranged between 0.119 and 0.256, HI titres were generally higher than 1:20 and VN titres were between 1:8 and 1:128. In contrast, BCoV antibodies were not detected in any control calves.

In order to detect potential amino acid (aa) residues involved in virus attenuation, the S gene sequences of the wild-type (438/06-TN) and cell-adapted (438/06-TN-50) strains were determined by RT-PCR amplification and subsequent sequencing. The S genes of both strains were 4092-nucleotide (nt) long. In comparison to the wild type, 10 nt changes were identified in the S gene of the attenuated virus, that caused 9 amino acid (aa) changes (Table 2). The encoded proteins were long 1363 aa and matched the best aa identity (98%) to the Korean BCoV strain KWD2 (Jeong *et al.*, 2005). No deletions or insertions were observed in the aa sequence of strain 438/06-TN-50 with respect to the original non cell-adapted virus.

TABLE 2 - *Substitutions in the nucleotide (nt) and amino acid (aa) sequences of the S gene of wild-type and cell-adapted 438/07-TN strain.*

Codon	*438/07-TN*		*438/07-TN-50*	
	nt	*aa*	*nt*	*aa*
24	CTT	I	CGT	R
36	ATT	I	ACT	T
39	GAT	D	GCT	A
115	AAT	N	AAG	K
179	CAA	Q	CGA	R
263	ACC	T	ACA	T
906	GAT	D	GCT	A
1223	GTT	V	TTT	F
1259	CCA	P	TCA	S
1341	AAA	K	ATA	I

Vaccination of cows to prevent BCoV-induced enteritis in newborn calves has being routinely carried out in cattle herds worldwide for some decades. Calves receiving maternally-derived antibodies (MDA) through colostrum are protected against the clinical forms of the disease in the first weeks of age (Crouch *et al.*, 2001). However, since MDA undergo a progressive decrease with the age of the animals, calves born to vaccinated dams are exposed to BCoV infection/disease when MDA reaches non-protective levels. Accordingly, those calves should be actively vaccinated at 2-3 months of age. However, to date only vaccines for protection against the intestinal form have been available on the market, as those formulations are prepared with enteric BCoV strains and also contain rotaviruses and *Escherichia coli* that are involved in the occurrence of neonatal diarrhoea. In order to overcome the limitations of existing vaccine formulations, we have developed a monovalent MLV vaccine prepared with a respiratory BCoV strain. The candidate vaccine was proven to be safe, as no adverse reactions were observed in newborn calves administered by the natural route of BCoV infection (oronasally) with a dose containing 10 times the viral titre which should be employed for routine vaccination. At the same time, the vaccine was able to elicit the production of high BCoV antibody titres in all vaccinated calves. The efficacy of the vaccine was not assessed as the vaccinates were not challenged, but based on the results of serology, a protection at least from the clinical form of disease would be expected. BCoV-induced BIRD is an emerging disease in cattle herds in several countries, including Italy (Decaro *et al.*, 2008a). The increasing rate of BCoV isolation from cattle with respiratory distress highlights the need for monovalent vaccines prepared with respiratory BCoV strains (not yet available on the market). The candidate vaccine should preferably be MLV as only attenuated strains may induce an active antibody- and cell-mediated immunity at the same time. Although the present report proved that MLV strain 438/06-TN-50 is safe and immunogenic, future challenge studies are required to assess the real level of protection induced in cattle by this attenuated strain against virulent BCoV infection.

The nucleotide sequence of the S protein genes of the wild-type and cell adapted BCoV strains have been deposited in GenBank under accession numbers 1102270 and 1102274.

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian Ministry of Health, Ricerca finalizzata 2007, project "Mammalian coronaviruses: molecular epidemiology, vaccine development and implications for animal and human health" and from University of Bari, Italy, Ricerca di Ateneo 2008, project "Patogeni emergenti dei ruminanti.

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