Field Trials on a Live Bovine Respiratory Syncytial Virus Vaccine in Calves

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ABSTRACT. Field trials were carried out in calves using a live bovine respiratory syncytial (BRS) virus vaccine prepared from the attenuated BRS virus, strain rs-52. Two hundred seventy-five and 353 calves were vaccinated intranasally and intramuscularly, respectively. No undesirable postvaccinal reactions were observed in the vaccinated calves. Of the serum neutralizing (SN) antibody negative calves 89.7% (26/29) and 92.8% (90/97) developed SN antibody 1 month after intranasal and intramuscular vaccination, respectively. Most of the calves having SN antibody titers of 1:1 or 1:2 at the time of vaccination showed a significant increase in SN antibody titer. About 70% and 90% of the calves vaccinated intranasally and intramuscularly, respectively, maintained SN antibody for 6 months after vaccination. In a field trial, a natural BRS virus infection occurred about 5 months after the start of the trial. Ten of the 16 unvaccinated control calves showed respiratory symptoms due to BRS virus infection. On the contrary, all of the 68 vaccinated calves exhibited no symptoms at all, indicating efficacy of the vaccine.—KEY WORDS: bovine respiratory syncytial virus, field trial, live vaccine.

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Several attenuated bovine respiratory syncytial (BRS) live virus vaccines for the prevention of the respiratory disease caused by BRS virus in cattle have been developed in other countries [1, 2, 4, 7–15]. Their immunogenicity is, however, weak, twice or thrice intramuscular injections are required and their application by intranasal inoculation has not been studied so far. Furthermore, all these attenuated viruses were not low temperature-adapted strain and produced by using the bovine cell cultures. In addition, a marker *in vitro* which enables to distinguish the attenuated strain from the wild type strain has not been fully investigated.

In the previous report [5], the authors established an attenuated strain of BRS virus, designated as rs-52 strain, by serial passages at low temperature (30°C) in HAL cells established from adult Syrian hamster lung. This strain had a marker *in vitro* which enables to clearly distinguish it from the virulent strain in virus growth curve at 30°C in HAL cells. In the laboratory experiments, calves inoculated intranasally or intramuscularly with a single dose of this strain showed no clinical signs and no virus excretion which was confirmed by neither virus recovery from nasal swabs nor antibody production in contact control animals. However, the inoculated calves produced serum neutralizing (SN) antibodies and acquired strong immunity against challenge with the virulent strain of BRS virus. These laboratory results suggest that this attenuated strain of BRS virus can be used as a live virus vaccine. To verify these laboratory findings, field trials were performed to determine the safety and efficacy of the vaccine prepared from the attenuated rs-52 strain for actual use in cattle.

MATERIALS AND METHODS

Virus: The attenuated rs-52 strain [5] of BRS virus was used for the preparation of vaccine. The NMK7 strain [3] of BRS virus was also used for the SN test.

Cell culture: HAL and Vero cells were used. The growth medium (GM) and maintenance medium (MM) for these cells were prepared as described previously [5].

Vaccine: HAL cells cultured in 5-litter rolling culture bottle were inoculated with the rs-52 strain (moi $\fallingdotseq$ 0.1). After virus adsorption at 37°C for 60 min, the culture was fed with 500 m*l* of MM and incubated for 10~14 days in a rolling state. Then, the culture fluid was harvested from the bottle and centrifuged at 3,000 rpm for 15 min. The resulting supernatant was used as the material of vaccine. The vaccine virus was mixed with an equal volume of a solution containing 10% lactose, 5% saccharose, 2% pepton, and 0.3% polyvinylpyrrolidon (molecular weight=700,000). Two m*l* of the mixtures were dispensed into 15-m*l* vials, freeze-dried, and sealed under reduced pressure. Before used, freeze-dried vaccine in a vial was reconstituted with 10 m*l* of dissolving medium (NaCl 8.0 g, $Na_2HPO_4 \cdot 2H_2O$ 0.45 g, $NaH_2PO_4 \cdot 12H_2O$ 2.52 g, and 1% phenol red 1 m*l* in purified water 1,000 m*l*; pH 7.2; sterilized at 115 °C for 20 min).

Virus titration: This was carried out in tube cultures of Vero cells by the method described previously [5]. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the method of Reed and Meunch [6].

SN tests: Serial twofold dilutions of serum inactivated by heating at 56°C for 30 min were made in GM. Each dilution was mixed with an equal volume of GM containing 200 $TCID_{50}$/0.1 m*l* of the NMK7 strain. The mixtures were kept at 22°C for 24 hr. Each mixture was inoculated into tube cultures of Vero cells by the method described previously [5]. SN antibody titer was expressed by the reciprocal of the highest serum dilution which showed neutralization in at least two of the four tubes. The serum was taken as antibody positive when it showed neutralization at 1:1 or higher serum dilution.

Field trials: Lots 3, 4, and 11 of live BRS virus vaccine showing a virus titer of $10^{3.5}$, $10^{4.5}$, and $10^{6.0}$ $TCID_{50}$/m*l*, respectively, were used. As shown in Table 1, 1 m*l* each of the vaccine was inoculated into 275 calves intranasally and 353 calves intramuscularly, and 143 calves were left without inoculation as unvaccinated control in 4 farms in different locations in Japan. All the calves were observed for general clinical symptoms throughout the experimental period. From some of the calves, blood samples were taken for measurement of SN antibody titer at the time of vaccination and after vaccination.

RESULTS

Safety of vaccine in calves: Lots 3 and 4 of vaccine were intranasally inoculated into 241 and 34 calves (275 calves in total), respectively. Lots 3, 4, and 11 of vaccine were intramuscularly inoculated into 249, 34, and 70 calves (353 calves in total), respectively. Then, general clinical symptoms were observed for 14 days after vaccination. As a result, undesirable postvaccinal reactions were not detected in these calves.

SN antibody responses one month after vaccination in SN antibody negative calves: All the calves used in this experiment had SN antibody titer of less than 1:1 at the time of vaccination. In the intranasal vaccination, 89.5% (17/19) of the calves vaccinated with Lot 3 and 90.0% (9/10) of the calves with Lot 4 developed SN antibody 1 month after vaccination. The geometric means (GM) of SN antibody titers increased to 4.95 and 2.14 in the calves vaccinated with Lots 3 and 4, respectively. In the intramuscular vaccination, 91.3% (21/23) of the calves vaccinated with Lot 3, 90.9% (10/11) of the calves with Lot 4, and 93.7% (59/63) with Lot 11 developed SN antibody. The GM antibody titers were 7.26, 4.51, and 5.01 in the calves vaccinated with Lots 3, 4, and 11, respectively. There was a tendency that SN antibody titer was higher in calves vaccinated intramuscularly than that in those vaccinated intra-

Table 1. Summary of field trials of live BRS virus vaccine

Vaccine tested		Farm	Calves used		Experimental	Number of calves
Lot No.	Dose[a)]	(Prefecture)	Breed	Age of month	group	tested
3	$10^{3.5}$	A (Ibaraki)	Holstein	About 1 month old	IN	241
					IM	249
					control	97
4	$10^{4.5}$	B (Hyogo)	Holstein	About 1~3 months old	IN	34
					IM	34
					Control	16
11	$10^{6.0}$	N and T (Osaka)	Holstein and Black Japanese	About 3~4 old months	IM	70
					Control	30
				Total	IN	275
					IM	353
					Control	143
				Grand total		771

a) $TCID_{50}$/m*l*.
b) IN: Intranasal vaccination, IM: Intramuscular vaccination, Control: Unvaccination.

Table 2. SN antibody responses one month after vaccination in SN antibody negative calves

Vaccine tested		Group[a)]	SN antibody response	
Lot No.	Dose ($TCID_{50}$/m*l*)		Number of positive/total (%)	Geometric mean titer (range)
3	$10^{3.5}$	IN	17/19(89.5)	4.95(<1~32)
		IM	21/23(91.3)	7.26(<1~32)
		Control	0/10 (0)	<1
4	$10^{4.5}$	IN	9/10(90.0)	2.14(<1~8)
		IM	10/11(90.9)	4.51(<1~16)
		Control	0/5 (0)	<1
11	$10^{6.0}$	IM	59/63(93.7)	5.01(<1~64)
		Control	0/26 (0)	<1
Total		IN	26/29(89.7)	3.71
		IM	90/97(92.8)	5.41
		Control	0/41 (0)	<1

a) In: Intranasal vaccination, IM: Intramuscular vaccination, Control: Unvaccination.

Table 3. SN antibody responses one month after vaccination in SN antibody positive calves

Vaccine tested		SN antibody titer at the time of vaccination	Number with 4-fold or greater increase/total (%)		
Lot No.	Dose ($TCID_{50}$/m*l*)		Group[a)] IN	IM	Control
3	$10^{3.5}$	1	3/6 (50.0)	4/5 (80.0)	0/4 (0)
		2	4/5 (80.0)	8/13(61.5)	0/1 (0)
		4	2/10(20.0)	2/5 (40.0)	0/2 (0)
		8	0/8 (0)	1/6 (16.7)	0/5 (0)
		Total	9/29(31.0)	15/29(51.7)	0/12(0)
4	$10^{4.5}$	2	0/2 (0)	3/4 (75.0)	0/1 (0)
		4	1/3 (33.3)	0/1 (0)	0/2 (0)
		8	0/4 (0)	0/3 (0)	0/1 (0)
		16	0/1 (0)	·	·
		Total	1/10(10.0)	3/8 (37.5)	0/4 (0)
11	$10^{6.0}$	1	·	1/1 (100)	·
		2	·	3/3 (100)	0/2 (0)
		4	·	0/2 (0)	0/2 (0)
		8	·	0/1 (0)	·
		Total	·	4/7 (57.1)	0/4 (0)
Total		1	3/6 (50.0)	5/6 (83.6)	0/4 (0)
		2	4/7 (57.1)	14/20(70.0)	0/4 (0)
		4	3/13(23.1)	2/8 (25.0)	0/6 (0)
		8	0/12 (0)	1/10(10.0)	0/6 (0)
		16	0/1 (0)	·	·
		Grand total	10/39(25.6)	22/44(50.0)	0/20(0)

a) IN: Intranasal vaccination, IM: Intramuscular vaccination, Control: Unvaccination.

Table 4. Persistence of SN antibody in the calves vaccinated with Lot 3 ($10^{3.5}$ TCID/m*l*) of live BRS virus vaccine

SN antibody at the time of vaccination	Group[a)]	Number of SN antibody positive/total (%)				Geometric mean SN antibody titer (range)			
		Months after vaccination				Months after vaccination			
		0	1	3	6	0	1	3	6
Negative	IN	0/19 (0)	17/19 (89.5)	17/19 (89.5)	14/19 (73.7)	<1	4.95 (<1~32)	3.98 (<1~16)	2.00 (<1~8)
	IM	0/23 (0)	21/23 (91.3)	22/23 (95.7)	20/23 (87.0)	<1	7.26 (<1~32)	7.26 (<1~≧64)	4.63 (<1~≧64)
	Control	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	<1	<1	<1	<1
Positive	IN	29/29 (100)	27/29 (93.1)	23/29 (79.3)	20/29 (69.0)	3.21 (1~8)	3.53 (<1~16)	2.37 (<1~≧64)	1.65 (<1~8)
	IM	29/29 (100)	27/29 (93.1)	28/29 (96.6)	27/29 (93.1)	2.66 (1~8)	6.89 (<1~32)	5.30 (<1~≧64)	4.59 (<1~16)
	Control	12/12 (100)	6/12 (50.0)	4/12 (33.3)	2/11 (18.2)	3.35 (1~8)	1.12 (<1~4)	0.75 (<1~4)	0.65 (<1~2)

a) IN: Intranasal vaccination, IM: Intramuscular vaccination, Control: Unvaccination.

nasally. On the other hand, none of the 41 unvaccinated control calves developed SN antibody (Table 2).

SN antibody responses one month after vaccination in SN antibody positive calves: The calves used in this experiment had SN antibody titers of 1:1~1:16 at the time of vaccination. Only 25.6% (10/39) of the total calves vaccinated intranasally with Lots 3 or 4 and 50.0% (22/44) of the total calves intramuscularly with Lots 3, 4 or 11 showed fourfold or greater increase in SN antibody titer 1 month after vaccination. In addition, more than 50% of the calves having SN antibody titers of 1:1 or 1:2 showed a remarkable rise in SN antibody titer. On the contrary, antibody production was considerably interfered in the calves having SN antibody titers of 1:4 or higher. On the other hand, the unvaccinated control calves showed no increase in SN antibody titer (Table 3).

Persistence of antibody in vaccinated calves: As shown in Table 4, in SN antibody negative calves, the GM antibody titer 1 month after intranasal vaccination with Lot 3 was 4.95 and declined to 2.00 by 6 months after vaccination, but 73.7% (14/19) of them were SN antibody positive. In the calves vaccinated intramuscularly, the GM antibody titer was 7.26 and 91.3% (21/23) of them produced SN antibody 1 month after vaccination. Even 6 months after vaccination, the GM antibody titer was 4.63 and 87.0% (20/23) of them were still positive. On the other hand, SN antibody titers of the unvaccinated control calves were always less than 1:1. Further, in SN antibody positive calves at the time of vaccination, the GM antibody titer of the calves vaccinated intranasally decreased from 3.21 to 1.65 six months after vaccination, but 69.0% (20/29) of them retained SN antibody. In the calves vaccinated intramuscularly, the GM antibody titer increased from 2.66 to 6.89 one month after the vaccination. Even 6 months after vaccination the titer was 4.59 and 93.1% (27/29) of the calves still retained SN antibody. In the unvaccinated control calves, the GM antibody titer declined remarkably and 81.8% (9/11) of them lost SN antibody until 6 months after the start of this test.

SN antibody and clinical responses following a natural BRS virus infection in the vaccinated calves: In the field trials using Lot 4, 10 of the 16 unvaccinated control calves showed respiratory symptoms such as pyrexia, cough and nasal discharge about 5 months after the start of this trial. On the other hand, none of the calves vaccinated intranasally or intramuscularly manifested abnormal clinical signs including respiratory symptoms. Furthermore, in the unvaccinated control calves, the antibody titers increased from less than 1:1 two months before the onset of the disease (3 months after vaccination) to 1:64 or higher 1 month after the onset (6 months after vaccination). On the contrary, almost the vaccinated calves with a SN antibody titer

Table 5. SN antibody and clinical responses following natural BRS virus infection in the calves vaccinated with Lot 4 ($10^{4.5}$ $TCID_{50}$/m*l*) of live BRS virus vaccine

Group[a]	SN antibody before BRS virus prevalence[b]	SN antibody response				Clinical response[d]	
		Pre[b]		Post[c]			
		Number of calves tested	Geometric mean titer (range)	Number of calves tested	Geometric mean titer (range)	Number of positive/total (%)	
IN	Negative	2	<1	1	≧64	0/2	(0)
	Positive	9	2.32(1~16)	7	7.32(1~≧64)	0/9	(0)
	Not tested		·		·	0/23	(0)
					Total	0/34	(0)
IM	Negative	1	<1	Not tested	·	0/1	(0)
	Positive	9	2.51(1~8)	9	9.26(1~≧64)	0/9	(0)
	Not tested		·		·	0/24	(0)
					Total	0/34	(0)
Control	Negative	4	<1	3	≧64	3/4	(75.0)
	Not tested		·		·	7/12	(58.3)
					Total	10/16	(62.5)

a) IN: Intranasal vaccination, IM: Intramuscular vaccination, Control: Unvaccination.
b) Three months after vaccination (about 2 months before BRS virus prevalence).
c) Six months after vaccination (about 1 month after BRS virus prevalence).
d) Respiratory symptoms such as fever, cough, and nasal discharge.

of 1:1~1:16 tended to show low-titer response compared with the unvaccinated control, although a few lost already SN antibody before the epizootic and showed a remarkable increase in SN antibody titer after the prevalence of the disease (Table 5). As mentioned above, most of these vaccinated calves showed a good SN antibody response 1 month after vaccination, although SN antibody production was interfered in the SN antibody positive calves at the time of vaccination. From these results, it was confirmed that the disease was caused by BRS virus and the vaccination protected against this disease.

DISCUSSION

As for live BRS virus vaccine, there have been many reports by foreign investigators [1, 2, 4, 7–15]. Zygraich and Wellemans [15] indicated that a single intramuscular inoculation with the vaccine containing $10^{5.3}$ or $10^{5.7}$ $TCID_{50}$ of virus produced SN antibodies in only about 30% and 80% of the vaccinated calves, respectively, and that 100% seroconversion was achieved only followed by twice intramuscular inoculation with the vaccine containing $10^{5.7}$ $TCID_{50}$ of virus. Verhoeff and Nieuwstadt [11] also reported using the same vaccine that antibody response in the calves was not enough by only a single intramuscular vaccination. In the fields, this vaccine has been used by twice or thrice intramuscular inoculations and appeared to be effective in reducing the incidence of respiratory disease due to BRS virus infection in the vaccinated calves [9–11]. But, this vaccine has not been used by intranasal inoculation because immunogenicity of the parent RB_{94} strain of the vaccine virus is remarkably low [12, 14] . The vaccine developed by Kucera *et al.* [4] has also been used by twice intramuscular inoculations in the field. Including these vaccines, all the vaccines developed by foreign investigators require twice or thrice intramuscular inoculations [1, 2, 4, 7–15] and are less labor-saving than our vaccine.

In the present study, three lots of live BRS virus vaccine, Lots, 3, 4, and 11 containing $10^{3.5}$, $10^{4.5}$, and $10^{6.0}$ $TCID_{50}$/m*l* of virus, respectively, were prepared tentatively from the attenuated rs-52 strain of BRS virus mentioned in the previous paper [5]. In the field trials, 1 m*l* each of these vaccines was intranasally or intramuscularly inoculated into calves. They manifested no abnormal clinical signs caused by vaccination and there were no contact infections in the cohabiting unvaccinated control calves. These results indicated that the vaccine was safe for calves raised in the fields. Other investiga-

tors have also reported the similar results obtained by intramuscular inoculation of live BRS virus vaccine [7–9, 11, 14, 15].

In the SN antibody negative calves vaccinated intranasally or intramuscularly, about 90% produced SN antibody 1 month after vaccination. In the field trials using Lot 3, 73.7% of the calves vaccinated intranasally and 87.0% of the calves intramuscularly maintained SN antibody even 6 months after vaccination. Therefore, there seems to be no problem in persistence of antibody.

Influences of maternal antibody on the vaccine effect have been observed with many live virus vaccines including live BRS virus vaccine [2, 11]. In the present field trials, some calves having SN antibody titers of 1:1~1:16 were present, probably because of the presence of maternal antibodies in the youngest calves. When these calves were vaccinated, a significant increase in SN antibody titer was observed at the high rate in the calves having SN antibody titers of 1:2 or lower, and at the low rate in the calves having SN antibody titers of 1:4 or higher. However, in the fields trials using Lot 3, disappearance of SN antibody was remarkably delayed in the vaccinated group compared with unvaccinated control group, suggesting an efficacy of vaccine.

In the field trials using Lot 4, a natural BRS virus infection was occurred about 5 months after the start of the trial. Most of the unvaccined control calves showed respiratory clinical signs associated with BRS virus infection and the SN antibody titers rapidly increased from less than 1:1 to 1:64 or higher after infection. In the intranasal and intramuscular vaccinated groups, none of the calves exhibited any clinical signs. Most of the vaccinated calves showed a slight increase in SN antibody titer. In a few calves of which SN antibody had been already lost at the time of infection, the antibodies became positive after infection. It seems likely that the protection for the disease in these calves is due to cell-mediated immune response or to a very low level SN antibody.

From the present study, it is concluded that the live BRS virus vaccine tentatively prepared from the attenuated rs-52 strain is safe when applied to calves in the fields and effective in the prevention of BRS virus infection, but the final evaluation of the effectiveness is to be waited for.

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