NOTE *Virology*

Field Application of the Combination of Neutralizing Test and Virus Isolation, So-called Spot Test, to Detect Herds Including Cattle Persistently Infected with Bovine Viral Diarrhea Virus

Yoshihisa SEKI[1)]*, Yukio M. SEIMIYA[1)], Masato MOTOKAWA[1)], Hiroshi MIYAZAKI[1)], Masaru KONNO[1)] and Gakuji YAEGASHI[1)]

[1)]*Iwate Prefecture Central Livestock Hygiene Service Center, 390–5 Sunagome, Takizawa-mura, Iwate 020–0173, Japan*

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ABSTRACT. To detect herds including cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV), application of the combination of neutralizing antibody detection and virus isolation, so-called spot test, were performed on sera of 3 calves selected from each of 26 farms. Nine farms were judged as positive because 64 or more antibody titers were detected from 2 or more calves or BVDV was isolated from one or more calves. PI cattle were detected from 8 of the 9 farms. The positive judgment on one farm was obtained only when the indicator virus used on the neutralizing test was genotypically identical with the isolate from the farm. These results suggest that the spot test can be effective in detecting herds with PI cattle and that the accuracy may be influenced by the genotypes of the indicator viruses.

KEY WORDS: bovine viral diarrhea virus, persistently infected cattle, spot test.

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Cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV), shedding large amounts of the virus in secretions and excretions throughout their lives, are the main source of the virus infections [1–3, 6]. Therefore, it is important for BVDV control to identify and remove PI cattle from herds. In several areas of Japan, the detection of BVDV gene from bulk milks (bulk milk test) has been utilized for the screening of dairy herds with PI cattle and followed by identification of many PI ones [9]. On the other hand, PI cattle are frequently dead or culled due to various diseased conditions prior to their adult age, such as mucosal disease (MD) and growth retardation [1–3]. These reports seem to suggest that many PI cattle are nulliparous, i.e., non-lactating. The bulk milk test can efficiently evaluate a dairy herd with 100 or more lactating cows by the examination of one sample, but cannot detect herds with only non-lactating PI cattle [9, 13], nor be applied to beef cattle herds without bulk milks.

Houe *et al.* [7] proposed another screening test on which it was considered a herd with PI cattle when high serum neutralizing antibody titers against BVDV were detected from 3 or more of 5 calves selected from the herd. The test is able to evaluate both of the herds with and without lactating PI cattle, but the presence of PI animals within selected calves may cause false-negative judgments since such animals develop no or low levels of the antibody titers [1, 3]. The authors [15] suggested on the basis of stochastics that this problem might be solved by the combination of neutralizing test and virus isolation using sera of 3 calves selected from each herd, so-called spot test. In this report, the results of field application of the spot test [15] to 26 farms in Iwate Prefecture are described to investigate accuracy of the test.

Virological and epidemiological examinations were conducted on 24 dairy farms (A-X) with 16 to 84 animals and 2 beef cattle farms (Y, Z) with 35 or 45 during the period from 2005 to 2006. Four cytopathogenic BVDV strains, Nose [8], IW21/98/CP, KS86-1CP [17] and KZ91CP [12], were prepared as the representative genotypes-1a, -1b, -1e and -2, respectively. The IW21/98/CP strain was isolated from a calf affected with MD in Iwate Prefecture in 1998.

For the spot test, the sera were obtained from 3 calves that were 6 to 12 months old, unvaccinated against BVDV and randomly selected from each of 26 farms, and subjected to the neutralizing test and virus isolation. The antibody titer was determined by the microtitration method [16] using 4 strains mentioned above as the indicator viruses, and the highest among values against each of the indicator viruses was regarded as the individual titer. Virus isolation was conducted by the interference test [16] with the Nose strain. Based on the previous criteria [15], the farm was judged as positive when 64 or more antibody titers were detected from 2 or more calves or when BVDV was isolated from 1 or more calves.

After the spot tests, sera from all 1,182 animals and leucocytes from 40 calves aged less than 3 months were obtained from 26 farms, and followed by BVDV isolation using the method mentioned above. The animal with isolation of the non-cytopathogenic virus was resampled at least 3 weeks later and identified as PI cattle when the virus was detected again [1].

The isolates recovered from PI cattle were phylogenically analyzed. The 5' untranslated region was amplified by RT-PCR using the 324 and 326 primers [20] and sequenced by the same primers. The nucleotide sequences were aligned

* CORRESPONDENCE TO: SEKI, Y., Iwate Prefecture Central Livestock Hygiene Service Center, 390–5 Sunagome, Takizawa-mura, Iwate 020–0173, Japan.
e-mail: yoshihisa-seki@pref.iwate.jp

by the CLUSTAL X method [19]. The phylogenetic tree was constructed using the neighbor-joining method [14] along with the corresponding sequences of IW21/98/CP and 13 other strains (No.12, Nose, IS8NCP/97, Bega, KS86-1NCP, 190CP, HKD858NCP/95, Osloss, SoCP/75, IS25CP/01, IS26NCP/01, KZ91CP, 890) obtained from DNA Data Bank of Japan.

Table 1 shows the virological findings. Application of the spot test to 26 farms disclosed 9 positive farms (A-G, Y, Z), 8 farms (A-E, G, Y, Z) judged by only antibody titers and other one farm (F) by both antibody titers and virus isolation. Subsequent virus isolation from all cattle in 26 farms resulted in detection of 12 PI cattle from 8 farms (A-F, Y, Z). There were plural PI animals in two farms (A, Y) and single PI one in 6 others. All 12 PI cattle had been raised in each farm after their birth. Out of 12 PI cattle the lactating dairy cow was limited to one 38-month-old animal from one farm (A). Eleven other PI cattle were 31 or less months old, including one 1-month-old calf from one farm (C), and nulliparous.

On the phylogenetic tree (the figure not shown), 5 isolates from 4 farms (A-D), 2 from 2 farms (E, F) and 5 others from 2 farms (Y, Z) were classified into genotypes-1a, -1c and -1b, respectively (Table 1).

On the spot tests, the antibody titers differed in various degrees due to the used indicator virus. The highest titers were obtained in 6 positive farms (A-D, Y, Z) when the indicator virus was genotypically identical with the isolate (gen-

Table 1. Results of the spot test for herd diagnosis as well as virus isolation from all cattle in 26 farms

Farm	No. of cattle in farms	Spot test						No. of PI cattle in farms	
		Selected calves	Serum neutralizing antibody titers				Virus isolation	Nulliparous	Lactating
			Nose (1a)[a)]	IW21/98/CP (1b)	KS86-1CP (1e)	KZ91CP (2)			
A	61	1	*256*[b)]	64	64	32	–	1 (31)[c)]	1 (38)[c)]
		2	*1024*	512	256	64	–		
		3	*2048*	512	512	256	–	1a[d)]	
B	35	1	*1024*	128	64	32	–	1 (4)	0
		2	*4096≤*	2048	2048	64	–		
		3	*4096≤*	2048	1024	128	–	1a	
C	55	1	*512*	256	16	32	–	1 (1)	0
		2	*1024*	512	128	64	–		
		3	*1024*	512	256	64	–	1a	
D	33	1	*512*	256	32	32	–	1 (21)	0
		2	*2048*	128	128	16	–		
		3	*2048*	512	64	32	–	1a	
E	38	1	<2	<2	<2	<2	–	1 (26)	0
		2	*1024*	512	256	256	–		
		3	*2048*	1024	1024	128	–	1c	
F	84	1	<2	<2	<2	<2	+	1 (12)	0
		2	*256*	128	8	4	–		
		3	*4096≤*	1024	128	64	–	1c	
G	37	1	<2	<2	<2	<2	–	0	0
		2	512	*2048*	256	256	–		
		3	1024	*4096≤*	512	128	–		
H	38	1	*4*	2	<2	<2	–	0	0
		2	*8*	*8*	<2	<2	–		
		3	*512*	*512*	*512*	128	–		
I–X	16–76	1	<2	<2	<2	<2	–	0	0
		2	<2	<2	<2	<2	–		
		3	<2	<2	<2	<2	–		
Y	35	1	256	*1024*	256	64	–	4 (2–5)	0
		2	2048	*4096≤*	512	128	–		
		3	2048	*4096≤*	512	256	–	1b	
Z	45	1	<2	<2	<2	<2	–	1 (2)	0
		2	16	*64*	8	4	–		
		3	64	*128*	16	32	–	1b	

a) The indicator virus with the genotype in parenthesis.
b) The italic value indicates the highest among antibody titers against each of 4 indicator viruses.
c) Numbers in parentheses represent the age in months of PI cattle.
d) The genotype of BVDV isolated from PI cattle in each farm.

otypes-1a or -1b) from each farm, in 2 positive farms (E, F) with the isolates of genotype-1c when the indicator virus was the genotype-1a strain, and in the remaining one farm (G) without isolates when the genotype-1b strain was used. Although 8 (A-G, Y) of 9 positive farms were judged as positive even when the indicator virus and isolate were genotypically different, the remaining one farm (Z) only when the genotypes of both viruses were identical. The calves from the latter farm (Z) possessed as relatively low antibody titers as 128 or less.

Epidemiological investigation of 2 farms (C, G) showed that a total of 53 cattle, 30 in the former (C) and 23 in the latter (G) farm, had been dead, culled or sold within 12 months prior to the spot test. One 21-month-old heifer in the former farm (C) had been culled due to growth retardation 4 months prior to the test, but no clinical manifestations suggesting MD or PI [1–3, 6] were observed in 52 others.

The present spot test, comprising neutralizing test and virus isolation, judged all of 8 farms (A-F, Y, Z) with PI cattle as positive and 17 (H-X) of 18 others without such animals as negative. The 8 farms with PI cattle consisted of 6 dairy and 2 beef cattle farms, and all PI animals in 7 of the 8 farms were nulliparous. The obtained results suggest that the spot test can predict with high accuracy both of the farms with and without lactating PI cattle, including beef cattle farms.

The highest neutralizing antibody titers are obtained when the indicator and infected viruses are genotypically identical [13]. On the present tests using several genotypes of indicator viruses, positive judgment on one farm (Z) was made only when both viruses were genotypically identical. The facts suggest that the genotype of the indicator virus would play an important role for exact evaluation of the antibody titers followed by accurate judgment on the spot test, since Japanese BVDV isolates have been classified into 7 genotypes (1a, 1b, 1c, 1e, 1f, So, 2), whose antigenicities are markedly different each other [4, 10–12, 18]. The isolates are frequently divided into genotypes-1a and -1b, occasionally genotypes-1c, -1e and -2 and rarely -1f and -So [4, 10–12, 18]. Therefore, it seems likely that the false-negative diagnosis on the spot test resulting from the antigenic differences between the infected and indicator viruses could be considerably avoided by using 5 major genotypes (1a, 1b, 1c, 1e, 2) as indicator viruses in Japan.

In 2 farms (E, F) with the isolates of genotype-1c, the highest antibody titers were observed when the indicator virus was the genotype-1a strain, since no genotype-1c strain was used. The results from the 2 farms seem to support the previous report [4, 11] suggesting that the genotype-1c strain is the most closely related in antigenicity to the genotype-1a among 4 strains (1a, 1b, 1e, 2) used in this study.

The false-negative diagnosis can be also made when the spot test was applied to the farm with the oldest PI animals aged 2 or 3 months, since no younger PI calves have enough time to disseminate the infections throughout the farm [5–7]. The present spot test evaluated one farm (C) with one 1-month-old PI calf to be positive. In the farm, one 21-month-old heifer with growth retardation had been fed until 4 months before. It seems likely that the heifer might have been a PI animal although devoid of the virological evidence, and that positive judgment of the farm might be a result influenced by the heifer, not by the PI calf.

The farms applied immediately after all PI cattle were eradicated can be judged as positive by the spot test [5–7]. One farm (G) without PI cattle was judged as positive. Although the exact reason remains unknown, PI cattle might have been included in 23 animals removed within the latest 12 months.

It seems that the present spot test could contribute to BVDV control as the primary screening test for beef cattle farms and as the secondary screening test for dairy farms judged as negative by the bulk milk test.

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