Longitudinal and cross-sectional detection of four bovine enteric viruses by multiplex-reverse transcription polymerase chain reaction: Identification of possible indicator viruses to assess biosecurity level at bovine farms

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ABSTRACT. It can be judged that if the detection frequency of prevalent pathogenic viruses decreases, biosecurity has been enhanced. To monitor bovine farm biosecurity levels, one-step multiplex reverse transcription polymerase chain reaction (RT-PCR) for the simultaneous detection of group A rotavirus (RVA), bovine torovirus (BToV), bovine enterovirus (BEV), and bovine coronavirus (BCV) was designed, with the aim of configuring candidates for “viral pathogen indicators”. A total of 322 bovine fecal samples were collected from calves aged less than three months at 48 bovine farms in Ibaraki and Chiba prefectures. At farm A, 20 calves were selected and sampled weekly for 12 weeks (184 samples); at farm B, 10 calves were selected and sampled for five weeks (50 samples); and at the rest of the 46 farms, 88 calves were sampled once. The screening on the 358 field samples proved positive for 27 RVA, 4 BToV, 55 BEV, and 52 BCV. In the successive sampling, RVA was detected once but not continuously, whereas BEV and BCV were detected in succession for up to five weeks. The results revealed that RVA was the primary agent among the positive samples obtained from calves aged three weeks or less, while BEV was the primary among those from the older than three weeks old. They can be employed as useful viral pathogen indicators for soundly evaluating biosecurity at bovine farms.

KEY WORDS: biosecurity, enterovirus, multiplex RT-PCR, rotavirus, viral pathogen indicator

For the safety of livestock products, the improvement and regulation of rearing hygiene regimes within livestock farms are important. The Ministry of Agriculture, Forestry and Fisheries (MAFF) in Japan established “the Standards of Rearing Hygiene Management” in the law of Act on Domestic Animal Infectious Diseases Control, in 2004 [11]. MAFF has been introducing the “hazard analysis critical control point (HACCP) at livestock farm level: Farm HACCP Standard” certification standard for livestock farms in Japan since MAFF announced this certification in 2009 [12]. For the farm HACCP certification, controlling biological hazards and enhancing biosecurity at the farm level are critical. Kim et al. reported that biosecurity procedures such as changing personal protective equipment (clothes and footwear) could prevent the transmission of porcine epidemic diarrhea (PED) virus to sentinel pigs [6]. It can be judged that if the detection frequency of prevalent viruses decreases, biosecurity has been enhanced.

Calf diarrhea is a multifactorial disease involving pathogens, environmental elements and management practices, hence the nature of this disease makes it hard to be effectively controlled in modern calf operations [2, 16]. The viral etiology of diarrhea, which causes considerable economic loss for bovine farms, includes group A rotavirus (RVA) [13], bovine torovirus (BToV) [8], bovine enterovirus (BEV) [1], and bovine coronavirus (BCV) [15]; hence, those viruses were selected as candidate “viral pathogen indicators”. Viral pathogen indicators within bovine farms in this study were tentatively defined as relatively highly prevalent viruses found at bovine farms in Japan. One-step multiplex reverse transcription polymerase chain reaction (RT-PCR) was used to detect these viruses to monitor the farms, as described by Fukuda et al. [5].
Thus, this study was performed to evaluate the outcome of farm HACCP, by trying to find and configure viral pathogen indicators for cattle farms.

**MATERIALS AND METHODS**

**Primer preparation**

Based on previous studies [1, 5, 10, 14, 15], four pairs of specific primers for one-step multiplex RT-PCR for the representative genes of the four viruses were designed: RVA, BToV, BEV, and BCV, as shown in Table 1. Each primer, set at 100 µM in Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA), was mixed at an equal volume and used as a primer mixture (final 10 µM for each primer).

**Sample collection**

A total of 48 cattle farms that kept calves of Japanese Black or crossbred cattle (Holstein × Japanese Black: F1) were selected for rectal feces sampling from September 2016 to February 2017. At farm A, located in Ibaraki Prefecture, 20 calves were numbered individually and sampled every 5 to 10 days for up to 12 weeks (total 220 samples). At farm B, located in Chiba Prefecture, 10 calves were numbered individually and sampled every week for up to five weeks (total 50 samples). At the rest of the 46 farms, located in Chiba Prefecture, one or two calves were randomly selected from each farm, and thereby 88 calves aged 1–13 weeks were sampled once. There was no invasive sampling of the calves.

**Isolation of viral RNAs**

One gram of feces was suspended in 4 ml of phosphate buffered saline, mixed well using a vortex mixer, and centrifuged at 12,000 × g for 3 min. Viral RNAs were extracted using ISOGEN-LS (Nippon Gene Co., Ltd., Tokyo, Japan), according to the manufacturer’s instructions. Just before use, 2 µl of RNA was mixed with 1 µl of dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) together with 4.5 µl of redistilled water (dH2O), and heated at 98°C for 5 min.

**One-step multiple RT-PCR**

To amplify viral RNAs, a PrimeScript™ One Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Kusatsu, Japan) was used according to the manufacturer’s instructions. Briefly, 7.5 µl of DMSO-treated viral RNA was mixed with 1 µl of PrimeScript 1 step Enzyme Mix, 12.5 µl of 2 × 1-Step Buffer, 0.8 µl of the primer mixture, and 3.2 µl of RNase-Free dH2O, totaling 25 µl and incubated at 50°C for 30 min, to make cDNA stop the reaction thereafter at 95°C, for 10 min. Subsequently, the PCR reaction was performed according to Fukuda et al. [5], with 35 cycles at 94°C for 45 sec, 52°C for 15 sec, and 72°C for 1 min, and a final incubation at 72°C for 10 min. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. These amplified products from one of each size, namely, 928 bp (RVA), 651 bp (BoTV), 483 bp (BEV), and 407 bp (BCV), were taken from the agarose gels, purified, and cloned into plasmid pCR® 2.1 vector, using a TA Cloning Kit (Thermo Fisher Scientific K. K., Tokyo, Japan). Each recombinant plasmid containing each amplified product was sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific K. K.) to confirm the virus specificity.

The sensitivity of one-step multiplex RT-PCR (containing four primer sets) was compared with one-step single RT-PCR (containing one primer set), using the recombinant plasmids as templates with a mixture of the four primer sets. For the multiplex RT-PCR, four plasmids were used as templates, while single RT-PCR was based on only one plasmid for each virus as a template. The plasmid copy number was determined by the molecular weight of the plasmid, and the DNA concentration was determined with the Nano Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific K. K.).

**Table 1. Primers used in the one-step multiplex RT-PCR**

| Virus          | Primer name          | Target gene  | Nucleotide 5’ to 3’(a)                                                                 | Product length (bp) | References |
|----------------|----------------------|--------------|----------------------------------------------------------------------------------------|---------------------|------------|
| Group A rotavirus (RVA) | GEN_VP6F             | VP6          | GGCTTTWAAACGAAGTCTTCGYYGTCATATTGGGTG       | 928                 | [10]       |
|                | GAR VP6_928R         |              | GAGAAAGAGGCAAGATGAAGTTACACGAGACACTCAACCA   | 651                 | [14]       |
| Bovine torovirus (BToV) | 1344-294             | Nucleocapsid | ACCTTTTGTAGCCGTTGTTTCCAAGATTGTA            | 483                 | [1]        |
| Bovine enterovirus (BEV) | BEV-F               | Polyprotein  | GATTAGCAGTTCACGCGGATTAGCAGCATTACCGACG     | 407                 | [15]       |

(a) W=A or T; Y=C or T.
RESULTS

Confirmation of the amplified product specificity and sensitivity of one-step multiplex RT-PCR

Preliminarily, samples from farm A were tested with one-step multiplex RT-PCR. Within 44 samples, bands corresponding to RVA, BToV, BEV, and BCV were amplified. The amplified products could be easily distinguished from each other following agarose gel electrophoresis (Fig. 1). Each representative amplified product for each virus was cloned into the plasmid, sequenced, and then compared with the published sequences. For RVA, the 928 bp product had homology at 96% with the RVA UK strain sequence of Accession No. X53667.1; for BToV, the 651 bp product had homology at 97% with the BToV Ishikawa/2010 Accession No. LC088094.1; for BEV, the 483 bp product had homology at 88% with the BEV strain Wye 8875 Accession No. AY724745.1; and for BCV, the 407 product had homology at 98% with the BCV E-AH65 Accession No. EF424615.1.

Using each of the four different recombinant plasmid DNAs containing each of our four different target viral genes as templates, the multiplex RT-PCR could amplify each of the specific target genes (Fig. 1). The detection limit of the multiplex RT-PCR was evaluated. As shown in Fig. 2, the single RT-PCR has 10–100 times higher sensitivity than the multiplex RT-PCR for detecting viral genes in plasmids, though the multiplex RT-PCR could detect $10^6$ to $10^4$ copies of plasmids. Non-specific extra bands that did...
Detection of viruses from field fecal samples

The four targeted viruses were detected in fecal samples. As shown in Fig. 3 and Tables 2 and 3, in some cases, two viruses were detected simultaneously in a given sample.

Table 2. Virus detection pattern at farm A

| Calf No. | Age by weeks |
|----------|-------------|
|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1        |   | R |  C |   | E | E | E | E | E | - | C | - |
| 2        | R |   | C |   | - | C | E | E | E |  E |   | - |
| 3        |   | C |   | C | C |   | C | E | E | C |   | - |
| 4        |   |   | R | C | E | E | E | E | E | C |   |  C |
| 5        | R |   |   |   | - | C |   |   |   |   | - | C | - |
| 6        |   | C |   |   | C |   | C | E | E | C |   |   |
| 7        |   |   |   |   | C | C | C | E | C | C |   |   |
| 8        |   |   |   |   | C | C |   | C | C |   |   |   |
| 9        |   |   |   |   | C | C |   | C | C |   |   |   |
| 10       |   | R |   | C |   |   | E | E |   |   |   | T |
| 11       | - | R | C | C | C | E | E | E | T | - |   | - |
| 12       | - | - | Died |
| 13       |   | R | C | E | E | E | E | E | C |  E | - |
| 14       |   |   |   |   |   | - | C | E | E | E | - |
| 15       |   |   |   |   |   | E | C | E | E | E |   | T |
| 16       |   | - | - | Died |
| 17       | R |   | C | E | E | E | E | - |   |   |   |
| 18       |   | R | - | C | E | E | C |   |   | - |   |
| 19       |   | - | - | - | C | E | C |   |   | C |   | - |
| 20       |   | - | C | - | E | C | E | C | C | - |   | - |

At farm A, located in Ibaraki Prefecture, 20 calves were numbered and sampled every 5 to 10 days for up to 12 weeks (total 220 samples). Calves No. 12 and 16 died at the ages of two and four weeks, respectively. R, group A rotavirus; E, bovine enterovirus; C, bovine coronavirus; T, bovine torovirus; “-”, not detected.

Table 3. Virus detection pattern at farm B

| Calf No. | Age by weeks |
|----------|-------------|
|          | 1 | 2 | 3 | 4 | 5 |
| 1        |   | R |   |   |   |
| 2        |   |   | R | - |   |
| 3        |   |   |   | - | - |
| 4        |   |   |   | T | - |
| 5        |   |   |   | R | C |
| 6        |   |   |   | R | C |
| 7        |   |   |   |   | - |
| 8        |   |   |   | C | - |
| 9        |   |   |   | R | C |
| 10       |   |   |   | R | E |

At farm B, located in Chiba Prefecture, 10 calves were numbered and sampled every week for up to five weeks (total 50 samples). No virus was detected in calves No. 3 and 7. R, group A rotavirus; E, bovine enterovirus; C, bovine coronavirus; T, bovine torovirus; “-”, not detected.

Detection of viruses from field fecal samples

The four targeted viruses were detected in fecal samples. As shown in Fig. 3 and Tables 2 and 3, in some cases, two viruses were detected simultaneously in a given sample.

Table 2 shows the virus detection pattern at farm A. At that farm, 20 calves were numbered and individually sampled every 5 to 10 days up to 12 weeks old. Calves No. 12 and 16 died at the ages of two and four weeks, respectively. From a total of 220 samples at farm A, RVA was detected in eight calves, of which seven were three weeks of age or less. BEV was detected in 17 of 20 calves aged six weeks or not match any size of the targeted genes appeared in 5% of samples, and were all less than 300 bp.
more. As shown in Table 2, BEV was detected up to five successive times in the same calves. BCV was detected in 17 of 20 calves. This virus was also detected five successive times in the same calves. BToV was detected in two calves aged over nine weeks.

Table 3 shows the virus detection pattern at farm B. Ten calves were numbered and sampled weekly at the ages of one to five weeks. RVA was detected in six calves, of which five were two to three weeks old. RVA was detected once in each calf up to five weeks old. BEV and BCV were also detected.

In Table 4, the results of 88 samples from the 46 farms were shown. RVA was detected in 13 samples, of which nine were aged three weeks or less. BEV was detected in three samples, of which two were aged six weeks or more. BCV and BToV were each detected once at the age of six weeks.

DISCUSSION

Nucleotide sequence analyses and comparisons of the obtained PCR products with their referenced viral strains demonstrated that one-step multiplex RT-PCR could amplify and detect four target viral genes belonging to the four representative viruses. A detection limit test showed that at least 10^6 copies of templates were needed to detect the target viral genes using this method. Fukada et al. showed that with their multiplex RT-PCR, the detection limit for bovine group B rotavirus was 10^6 copies/ml, and that the sensitivity of one-step multiplex RT-PCR toward RVA was lessened compared to that toward BCV and BToV [5]. To the best of our knowledge, there is no available data on the RVA copy number that could be compared to our data. The specificity test showed that our method had the ability to detect four specific target viruses simultaneously. However, our data also showed that the detection of RVA was less sensitive (10–100 times) than that of BCV, BEV, and BToV.

In the field sampling, we randomly sampled rectal feces from calves under normal conditions. The screening of the 358 fecal samples proved positive for 27 RVA, 4 BToV, 55 BEV, and 52 BCV. In the successive sampling at farm A, RVA was detected once in each calf, but not continuously. In comparison, BEV and BCV were detected in succession for up to five weeks in the same calves.

As reported by Cho and Yoon [2], the RVA-infected calves secreted large amounts of the virus in their feces for five to seven days. Koopmans et al. [7] reported that BToV was detected in feces for four days. On the contrary, Chrouch et al. [4] reported that BCV was shed from infected calves for a period of 12 weeks. Cliver and Bohl [3] demonstrated BEV shedding for more than 16 weeks. They also reported that BEV was not recovered in calves aged less than four weeks.

Our present results revealed that RVA was the primary agent among the positive samples obtained at the age of three weeks or below, while BEV was the primary among those obtained at more than three weeks of age. Lay et al. reported that BEV was found in feces from 76% of cattle and suggested BEV as an indicator of fecal contamination [9]. Together with our findings, RVA and BEV can be referred to as reliable viral pathogen indicators for evaluating biosecurity at bovine farms.

PED is a highly contagious viral disease; however, proper biosecurity procedures, such as changing personal protective equipment (clothes and footwear), could prevent its transmission [6]. In this report, taking a shower was not reported to be essential for controlling PED virus transmission. Normally, at bovine farms, showering-in is not popular. It is possible to increase the level of biosecurity at bovine farms through the farm HACCP approach by changing boots and clothing, the appropriate use of disinfectants, or limiting access to the farms. In such cases, the presence and detection of the prevalent indicator viruses will be decreased.

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