

Molecular Epidemiology and Genotyping of Bovine Viral Diarrhea Virus in Xinjiang Uygur Autonomous Region of China

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Abstract—As part of national epidemiological survey on bovine viral diarrhea virus (BVDV), a total of 274 dejecta samples were collected from 14 cattle farms in 8 areas of Xinjiang Uygur Autonomous Region in northwestern China. Total RNA was extracted from each sample, and 5'-untranslated region (UTR) of BVDV genome was amplified by using two-step reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR products were subsequently sequenced to study the genetic variations of BVDV in these areas. Among the 274 samples, 33 samples were found virus-positive. According to sequence analysis of the PCR products, the 33 samples could be arranged into 16 groups. All the sequences, however, were highly conserved with BVDV Osloss strains. The virus possessed these sequences belonged to BVDV-1b subtype by phylogenetic analysis. Based on these data, we established a typing tree for BVDV in these areas. Our results suggested that BVDV-1b was a predominant subgenotype in northwestern China and no correlation between the genetic and geographical distances could be observed above the farm level.

Keywords—bovine viral diarrhea virus, molecular epidemiology, phylogenetic analysis.

I. INTRODUCTION

BOVINE viral diarrhea virus (BVDV) is distributed in cattle and causes significant economic losses worldwide. BVDV was first isolated in China from a stud stock that imported from New Zealand, and spread from the northeast and northern China to the northwest China [1]-[3]. Early epidemiologic survey for BVDV had shown that the average positive ratio in China was 19.15% [4] and two virus species, BVDV-1 and BVDV-2, were reported [5]. In general, adult cattle infected with BVDV didn't show apparent clinical signs [6]. While cattle serves as a natural host, BVDV-1 and BVDV-2 strains may also infect sheep, goats, wild ruminants and pigs [7], [8].

This research was funded by grants from the National Basic Research Program of China (973 Program) (2010CB530203).

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Recently, researchers in China had reported that the cow positive ratio in Shihezi area of Xinjiang region was 39.06% from collected blood samples [9]. The incidences of BVDV infection kept increasing for no effective eradication program was implemented systematically on regional or national levels. Measures to control this virus may include maintenance of closed herds, quarantine of animals to curb spread between herds, and identification and elimination of infected animals from herds [10]. Although vaccination has been used as a tool to lessen the impact of infection, there was no perfect vaccine since no single BVDV vaccine gave complete fetal protection [11], [12]. The vaccine may be inadequate in conferring herd-wide protection against the acute disease and in providing fetal protection against the development of persistently infected animals. Genetic diversification of BVDV was found to be the main cause of vaccination failure. The development of effective strategies to control BVDV infection relies on the knowledge of the type of strains present and the epidemiological profiles of the infections they cause [13], [14]. Unfortunately, the precise genomic region(s) and subgroup assignment of BVDV isolates in China have not been determined yet. Typing of BVDV into sub-genotypes is important for classification and for molecular epidemiology of BVDV [12]. Most of the phylogenetic analyses of the BVDV have been based on the 5'-UTR of the viral genome since it is the most conserved region of the pestivirus genome and fewer mutations are involved in the mathematic algorithm of the phylogenetic analysis [15]-[22]. The purpose of this study was to examine BVDV distributions in Xinjiang region of China and to map a typing tree in order to trace the routes of the infection and to survey the national BVDV epidemic status.

II. MATERIALS AND METHODS

A. Sample Collection

A total of 274 samples of dejecta from 14 cattle farms in 8 different areas in Xinjiang of China were collected into centrifuge tubes by cotton swab and stored at -20°C until using. The samples included those from sick cattle suffering diarrhea or abortion and those from healthy cattle. Normal saline containing penicillin and streptomycin was added to the sample in each centrifuge tube. The centrifuge tube was vortexed and then the tubes were incubated at 4°C for 12h in order to degerm.

The tubes were centrifuged at 10000rpm at 4 degree for 15min and the supernatant was transferred for viral RNA extraction.

B. Viral RNA Extraction and RT-PCR

RNA was extracted by Trizol reagent (Invitrogen, USA) according to the standard protocol. A two-step RT-PCR was carried out using a reverse transcription system (ImProm-II™ Reverse Transcription System, Promega). A pair of primers was used in the RT-PCR to amplify a 267 bp of the 5'-UTR gene (nt 108-375). The primer sequences were as follows:

P1:5'-CCTAGCCATGCCCTTAGTAGGACT-3' ,
P2:5'-GGAAGTCCATGTGCCATGTACA-3'.

The first-strand cDNA was synthesized in a 20µl mixture containing 1× RT buffer (10mM Tris-HCl, 50mM KCl, 0.1% Triton X-100, 25mM MgCl₂, pH 8.8), µM each primer, 50–100ng template RNA, 10 U recombinant RNasin ribonuclease inhibitor, 1mM dNTPs and 2 U M-MLV reverse transcriptase. The reaction mixture was incubated at 37°C for 1h followed by inactivation at 99 °C for 5min before being chilled immediately on ice for 5min. During the incubation on ice, a PCR mixture containing 1×RT buffer(contain MgCl₂), 0.5µM each primer, 2.5 U of Taq DNA polymerase (Tian Gen, China) and 10µl of cDNA mixture was prepared to a final volume of 50µl. The final RT-PCR mixture was then subjected to 45 amplification cycles; denaturation at 94°C for 1min, primer annealing at 55°C for 1min and extension at 68°C for 2min. A final extension was done at 68°C for 10min. PCR products were visualized by ethidium bromide staining after electrophoresis on 2% agarose gel.

C. Cloning and Sequencing

The PCR products were purified from low melting agarose gel using the Biotech Gel Extraction Kit according to the manufacturer's instructions. The purified PCR fragments were ligated into pMD18-T vector using T4 DNA ligase. Competent *E. coli* cells DH5α were transformed, screened and multiplied according to standard protocols. Multiplied bacteria containing the amplicons were sent for sequencing by Sangon biotechnology service limited company.

D. Phylogenetic Analysis

The nucleotide sequences of these PCR products were compared with NCBI blast and aligned using Clustal W multiple alignment method. To better fit the data in the program, we shortened all sequences to the 226-228bp fragment which was common to all isolates. A phylogenetic tree was constructed by the neighbor-joining method using the MEGA program, version 4.0, with 1,000 bootstrap replications. Genetic distance was also calculated using the MEGA program, version 4.0. Additional sequences representative of each known strain of BVDV-1, BVDV-2, border disease virus and classical swine fever virus were obtained from the GenBank and included in phylogenetic analysis. Previously reported BVDV isolates in China were included for comparison.

III. RESULTS

We isolated total RNA from 274 samples and proceeded to amplify 5'-untranslated region (UTR) of BVDV genome using RT-PCR [23]. We found that 33 samples were BVDV positive. Sequencing analysis showed 16 variants in the 33 5'-untranslated region (UTR) of BVDV genome. Based on the 16 different 5'-UTR sequences, a phylogenetic tree was built up (Fig. 1).

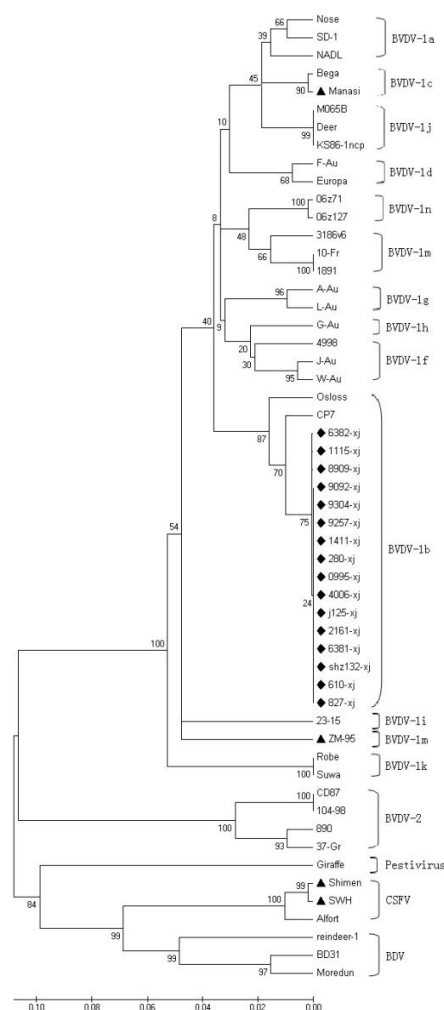


Fig. 1 Genetic typing of selected Pestivirus isolates in the 5'-UTR region

The sequences obtained in this work are labeled in filled rhombus. Chinese isolates are labeled in filled triangle. Sequences for other strains were acquired from the NCBI GenBank with the following accession numbers: Nose, AB078951; SD-1, M96751; NADL, M31182; Bega, AF049221; Manasi, EU159702; M065B, U97409; Deer, AB040132; KS86-1ncp, AB078950; F-Au, AF298065; Europe, AB000898; 06z71, DQ973181; 06z127, DQ973182; 3186V6, AF298062; 10-Fr, AF298054; 1891, AJ304384; A-Au, AF298064; L-Au, AF298069; G-Au, AF298066; 4998, AJ304385; J-Au, AF298067; W-Au, AF298073; Osloss, M96687; CP7, U63497; 23-15, AF298059; ZM-95, AF526381; Robe, AF299317; Suwa, AF117699; CD87, L32887; 104-98, AJ304381; 890, U18059; 37-Gr, EU327594; Giraffe, AB040131; Shimen, AF092448; SWH, DQ127910; Alfort, J04358; Reindeer-1, AF144618; BD31, U41112; Moredun, U65022. Genotype and subgenotype groupings are indicated on the right

It indicated all sequences in our collection showed high homology with Osloss strains at the nucleotide level and therefore phylogenetically clustered as BVDV-1b subtypes (Fig. 1). This was supported by a high bootstrap value (87%). Though Manasi strain isolated in this area shared higher sequence homology with Bega strain that was belonging to BVDV-1c subtypes, and another Chinese isolate named ZM-95 which was tentatively assigned as BVDV-1m by Jackva et al. were reported previously, we didn't find these strains this time.

For eight different areas, Kuytun area had shown highest positive ratio (22.2%) than others. The three areas of Korla, Akesu, Kashi had no positive samples in our survey. The positive ratio of calves was higher than the cow's. A number of 35 bovine appeared diarrhea had 31.4% positive ratio and others with non particular disease symptoms also reached 9.2%. (Table I).

TABLE I
DISTRIBUTION OF POSITIVE SAMPLES ORIGINATING FROM 8 AREAS

	Total	Positive	Positive rate (%)
Shihezi	62	8	12.9
Manasi	50	6	12.0
Shawan	49	7	14.3
Kuytun	45	10	22.2
Korla	18	0	0
Akesu	15	0	0
Kashi	20	0	0
Lli	15	2	13.3
Calves ^a	109	22	20.2
Cow ^b	165	11	6.7
Diarrhea	35	11	31.4
No diarrhea	239	22	9.2

IV. DISCUSSION

In this study, we collected 274 samples representative of cattle in Xinjiang area of the northwestern China. We used original non-cultivated clinical dejecta samples to avoid possible contamination of samples during cell culture. We then performed conventional RT-PCR using carefully designed primers to identify pestivirus RNA. We amplified a 235 bp amplicon for sequencing and the sequences obtained directly from gel-purified PCR products showed the presence of one viral strain in each sample. Several regions of the BVDV genome have also been sequenced for virus classification by several investigators [16], [24], [25]. However, the precise genomic region for subgroup assignment of BVDV isolates has not been determined yet. In this study, phylogenetic analysis of the 5'-UTR sequence was made of about 226-228 bp after data fitting in the program. We selected to analyze the 5'-UTR since it is the most conserved region of the pestivirus genome and it has fewer mutations involved in the mathematic algorithm of the phylogenetic analysis. This leads to trees with lower bootstrap values for individual branches [18], [19]. Our results show that the main subgroup of BVDV-1 circulating in Xinjiang is BVDV-1b. Genetic typing of BVDV-2 isolates has not been identified in current investigation, probably due to the small sample collection even though one BVDV-2 isolate had already been reported in Xinjiang [26].

This study genetically analyzed 33 positive samples from 14 farms in 8 regions of Xinjiang. High homology was observed

among positive samples detected in most farms. As regards with the geographic distribution, most of the positive samples were found in Shihezi, Manasi, Kuytun, and Shawan. These four areas were located not far away from each other. In fact, these were typical areas where BVDV infected previously due to immunotolerance. Samples were selected on the basis of the viral present in immunotolerant animals while variants are disadvantaged due to the ability of immunotolerant and immunocompetent animals to react and eliminate them after a transient infection. Up to now, we haven't rigorous quarantine measurement to aim directly at BVDV. It was likely that import of living animals was responsible for the introduction of the virus to these areas. This might explain why the positive ratio was so high. Regrettably, we did not detect any positive samples in Korla, Akesu, Kashi since there was MD outbreaks previously. That may attribute to fewer samples from these areas.

More recent analysis has revealed at least 13 BVDV-1 genetic groups (BVDV1a-1m) indicating considerable genetic diversity within this pestivirus genotype [27]. BVDV-1a and 1b are predominant subgroups in Ireland, the United Kingdom, Spain, India, Canada and the America [22], [28]-[32]. In contrast BVDV-1d and 1f dominate in some European countries [33], [34]. BVDV-1c originally thought to be unique to Germany was possibly transferred to Spain by animal export [35]. Another subgroup, BVDV-1g, is restricted to an area of middle Europe [33]. Generally speaking, BVDV-1 vaccine strains have been historically contained only BVDV-1a and BVDV-1b isolates [36]. Preliminary analysis has suggested that cells infected with a virus strain belonging to a particular BVDV-1 subgenotype were incompletely recognized by antibodies prepared even against a strain of the same subtype, confirming antigenic differences [22]. More definitive cross-protection studies are needed to address the importance of the epitopic diversity and determine whether future vaccines should be a mixture of several subgenotypes.

Because BVDV induces immunosuppression, resulting in secondary or opportunistic infection, it is difficult to clarify the relationships between BVDV1 subgenotypes and clinical manifestations [22]. In our study, we didn't find any correlation with MD outbreaks and other symptoms of non-symptomatic forms from different subgroup viruses despite the fact that some correlation had been observed in previous studies [37], [38].

In China, several BVDV isolates were obtained: CC184, ZM-95 [39], BVDV isolate [40], HN-1, HN-2 [41] and the other three isolates Manasi, letuyi, shihezi148 [42], [43] which were originated from Xinjiang and shown 100% homology in 5'-UTR with each other. It is worth pointing out that there haven't enough reports on phylogenetic and genotypic characterization of Chinese isolates. Therefore, we cannot obtain a general picture of BVDV prevalence in China. Hence, it will be valuable to continue to characterize more isolates from different regions of China as well as from neighboring countries to clarify epidemiological patterns in that area. These attempts will help understand global epidemiology of BVDV.

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