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Three clusters of bovine kobuvirus isolated in Korea, 2008–2010

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Abstract Fecal samples ($n = 107$) were collected from cattle with ascertained or suspected diarrheal disease on Korean farms during 2008–2010. Of these, 37 samples tested positive for bovine kobuvirus. The 37 positive samples came from 32 cattle that exhibited diarrhea and five cattle that were non-diarrhetic. The majority of the virus-positive feces samples were from calves under 1 month of age ($n = 25$). Nine of the 37 cattle infected with bovine kobuvirus were confirmed to have a co-infection with other viruses including bovine rotavirus ($n = 3$), bovine coronavirus ($n = 1$), bovine viral diarrhea virus ($n = 1$), and both bovine coronavirus and bovine viral diarrhea virus ($n = 4$). A neighbor-joining tree grouped 36 of the Korean kobuvirus strains (with the exception of the KB8 strain) into three clusters (G1, G3, and G4), while strains derived from Thailand and Japan (except the U1 strain) were included in the G2 cluster. The results indicated that Korean bovine kobuvirus has diverse lineages regardless of disease status and species.

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Picornaviruses are non-enveloped, single-stranded RNA viruses with a positive sense polarity. They cause a variety of important diseases in humans and animals [1]. The genus *Kobuvirus* (family Picornaviridae) includes genome ranging in length from 8.2 to 8.3 kb. The polyprotein of kobuvirus is cleaved post-translationally into three structural viral proteins (VP0, VP1, and VP3, with VP0 apparently not being cleaved further) and seven nonstructural proteins (2A–2C and 3A–3D). Kobuviruses encompass two officially recognized species, Aichi virus and bovine kobuvirus, as well as one candidate species, porcine kobuvirus [2–4]. Aichi virus was first isolated in 1991 from a person with acute gastroenteritis in Japan [2]. Bovine kobuvirus (strain U-1) was detected from serum and fecal samples of clinically healthy cattle in Japan [3]. It was subsequently isolated from cattle fecal samples in Thailand [5], Hungary [6], and Belgium [7]. In addition, bovine kobuvirus has been recently detected in domestic sheep in Hungary [8].

This study was performed to further characterize Korean bovine kobuvirus and to analyze the phylogenetic relationship between Korean strains and strains of other origin.

A total of 107 fecal samples were collected from cattle with ascertained or suspected diarrheal disease at 107 farms throughout Korea from January 2008 to July 2010. The cattle included 61 calves under the age of 30 days and 46 cows over the age of 30 days; Out of the total 107 fecal samples, 86 were from animals with diarrhea and 21 from non-diarrheic animals. The cattle were composed of 78 Korean cattle and 29 Holstein cattle. Samples were collected from the provinces of Gyonggi ($n = 32$), Gangwon ($n = 8$), Chungbuk ($n = 11$), Chungnam ($n = 42$),

Table 1 Summary of bovine kobuvirus strains

Strain	Animal	Age[a]	Year of isolation	Sample	Country	Province	Co-infection with other pathogens[b]	Accession no.
KB1	Korean native cattle	20 D	2008	Diarrhea	South Korea	Gangwon	BRV	HQ650164
KB2	Korean native cattle	1 M	2008	Diarrhea	South Korea	Gangwon	–	HQ650165
KB8	Korean native cattle	25 D	2008	Diarrhea	South Korea	Chungbuk	BRV	HQ650166
KB9	Korean native cattle	25 D	2008	Diarrhea	South Korea	Chungbuk	–	HQ650167
KB12	Korean native cattle	14 D	2009	Diarrhea	South Korea	Gyonggi	–	HQ650168
KB14	Korean native cattle	2 M	2008	Diarrhea	South Korea	Gyonggi	BCV, BVDV	HQ650169
KBH17	Holstein	15 D	2009	Diarrhea	South Korea	Chungnam	–	HQ650170
KB18	Korean native cattle	14 D	2009	Diarrhea	South Korea	Chungnam	BRV	HQ650171
KB31	Korean native cattle	14 D	2010	Diarrhea	South Korea	Chungnam	BCV , BVDV	HQ650172
KB34	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650173
KB38	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650174
KB39	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650175
KB40	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650176
KB41	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650177
KB42	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650178
KB45	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650179
KB55	Korean native cattle	3 M	2008	Diarrhea	South Korea	Gyonggi	–	HQ650180
KB59	Korean native cattle	3 M	2008	Diarrhea	South Korea	Gyonggi	–	HQ650181
KB60	Korean native cattle	3 M	2008	Diarrhea	South Korea	Chungbuk	–	HQ650182
KB61	Korean native cattle	1 D	2008	Non-diarrhea	South Korea	Gyonggi	–	HQ650183
KBH62	Holstein	1 Y	2009	Diarrhea	South Korea	Gyonggi	–	HQ650184
KB65	Korean native cattle	3 M	2008	Diarrhea	South Korea	Chungnam	BCV, BVDV	HQ650185
KBH67	Holstein	1 M	2008	Diarrhea	South Korea	Chungnam	BCV, BVDV	HQ650186
KBH71	Holstein	1 M	2008	Diarrhea	South Korea	Chungnam	–	HQ650187
KBH76	Holstein	15 D	2009	Diarrhea	South Korea	Chungnam	–	HQ650188
KB83	Korean native cattle	1 Y	2009	Diarrhea	South Korea	Gyonggi	BCV	HQ650189
KB85	Korean native cattle	6 D	2009	Non-diarrhea	South Korea	Gyongnam	–	HQ650190
KB86	Korean native cattle	2 Y	2009	Non-diarrhea	South Korea	Gyongbuk	BVDV	HQ650191
KB87	Korean native cattle	2 Y	2009	Non-diarrhea	South Korea	Gyongbuk	–	HQ650192
KBH92	Holstein	4 M	2009	Diarrhea	South Korea	Gyonggi	–	HQ650193
KBH94	Holstein	4 M	2009	Diarrhea	South Korea	Gyonggi	–	HQ650194
KB95	Korean native cattle	4 Y	2009	Non-diarrhea	South Korea	Gyongbuk	–	HQ650195
KB96	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650196
KB101	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650197
KB102	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650198
KB104	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650199
KB106	Korean native cattle	1 M	2010	Diarrhea	South Korea	Chungnam	–	HQ650200
CMB02	Cattle	7–49 D	2001–2004	Stool	Thailand		NT	EF659450
CMB03	Cattle	7–49 D	2001–2004	Stool	Thailand		NT	EF659451
CMB06	Cattle	7–49 D	2001–2004	Stool	Thailand		NT	EF659452
CMB07	Cattle	7–49 D	2001–2004	Stool	Thailand		NT	EF659453
CMB10	Cattle	7–49 D	2001–2004	Stool	Thailand		NT	EF659454
CMB11	Cattle	7–49 D	2001–2004	Stool	Thailand		NT	EF659455
Z20	Cattle	1 Y	2002	Stool	Hungary		NT	FJ225406
N2	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097162
N5	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097163
K3	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097152

Table 1 continued

Strain	Animal	Age[a]	Year of isolation	Sample	Country	Province	Co-infection withother pathogens[b]	Accession no.
K4	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097153
K6	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097154
K35	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097155
K36	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097156
K38	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097157
K44	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097158
K49	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097159
K55	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097160
K60	Cattle	2–4 Y	2003	Stool	Japan		NT	AB097161
U1	Cattle		2003		Japan		NT	NC_004421

[a] *D* day, *M* month, *Y* year

[b] *BRV* bovine rotavirus, *BCV* bovine coronavirus, *BVDV* bovine viral diarrhea virus, – no infection, *NT* not tested

Gyongbuk ($n = 10$), and Gyongnam ($n = 4$). Viral RNA was extracted from feces using TRIzol LS[b] according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Bovine kobuvirus was detected from fecal samples using reverse-transcript-polymerase chain reaction (RT-PCR) as previously described [3]. Oligonucleotide primers were designed based on the genome sequence of the U-1 strain (Accession No. AB084788) and have the following sequences: U-1f (sense, 5′-CATGCTCCTCGGTGGTCT CA-3′; nt 7,357) and U-1r (antisense, 5′-GTCCGGGTC CATCACAGGGT-3′; nt 7,987). Together, these primers amplify a 631-bp region of the 3D protein. PCR products of size 631 bp were visualized by electrophoresis and were cloned using the pGEM-T Vector System II (Promega, Madison, WI, USA). The cloned genes (three per sample) were sequenced, using T7 and SP6 promoter-specific primers, with an ABI Prism® 3730xl DNA Sequencer (Applied Biosystems, Foster City, CA, USA) at the Macrogen Institute (Macrogen, Seoul, Korea).

To investigate the relationship between kobuvirus and other bovine viruses that cause diarrhea in cattle, a screening test was conducted using primers specific for the detection of bovine rotavirus (BRV) [9], bovine coronavirus (BCV) [10], and bovine viral diarrhea virus (BVDV) [11], as previously described. Reverse transcription for the extracted RNA was performed using a cDNA synthesis kit (TaKaRa) and random hexanucleotide primers. The RT-PCR was run according to the following temperature–time profile: 42°C for 30 min, then 94°C for 5 min, followed by 35 cycles of virus-specific conditions, as follows: BRV: 94°C for 30 s, 54°C for 1 min and 72°C for 1 min; BCV: 94°C for 1 min, 58°C for 1 min and 72°C for 2 min; and BVDV: 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. For all viruses, the 35 denaturation–annealing–extension cycles were followed by a final extension at 72°C for 10 min. The resulting amplicon sizes were 309 bp for BRV, 730 bp for BCV, and 288 bp for BVDV. The sizes were assessed by 1% agarose gel electrophoresis and confirmed through the sequencing and analysis of the nucleotide sequence of each amplicon.

The nucleotide sequences of the Korean bovine kobuviruses were compared to those of kobuvirus reference strains in the GenBank database by BLAST. The nucleotide sequences were aligned using the Clustal W 1.8x program [12] and.aln files were generated. The.aln files were then converted to.meg files using Mega 4 [13] and a neighbor-joining tree was constructed (bootstrap replicates = 1,000) using the Kimura 2 parameter method for pairwise deletion at uniform rates. The nucleotide sequences of Korean bovine kobuvirus strains were deposited in GenBank as accession numbers HQ650164–HQ650200 (Table 1).

In prior studies, bovine kobuvirus was detected in 12 of 72 (16.7%) stool samples in Japan [3], 6 of 72 (8.3%) fecal samples in Thailand [5], and 2 of 32 (6.25%) fecal samples in Hungary [6]. Korean bovine kobuvirus was markedly more prevalent, being detected in 37 of the 107 (34.6%) fecal samples. Furthermore, the yearly frequency of the Korean positive samples was constant: $n = 12$ in both 2008 and 2009, and $n = 13$ in 2010 (Table 1). Thirty-two of the 86 diarrhea samples (37.2%) contained kobuvirus, compared with 5 out of 21 non-diarrhea samples (23.8%). However, this result cannot be taken as evidence of a causal relationship between kobuvirus infection and diarrhea, and such a causal relationship has been questioned in previous analyses [3, 5–7].

Infection by kobuvirus occurred in 38.5% (30 of 78) of the Korean native cattle and 24.1% (7 of 29) of the Holstein cattle. This result indicated that kobuvirus infection is not restricted to a single cattle species. Regarding the age

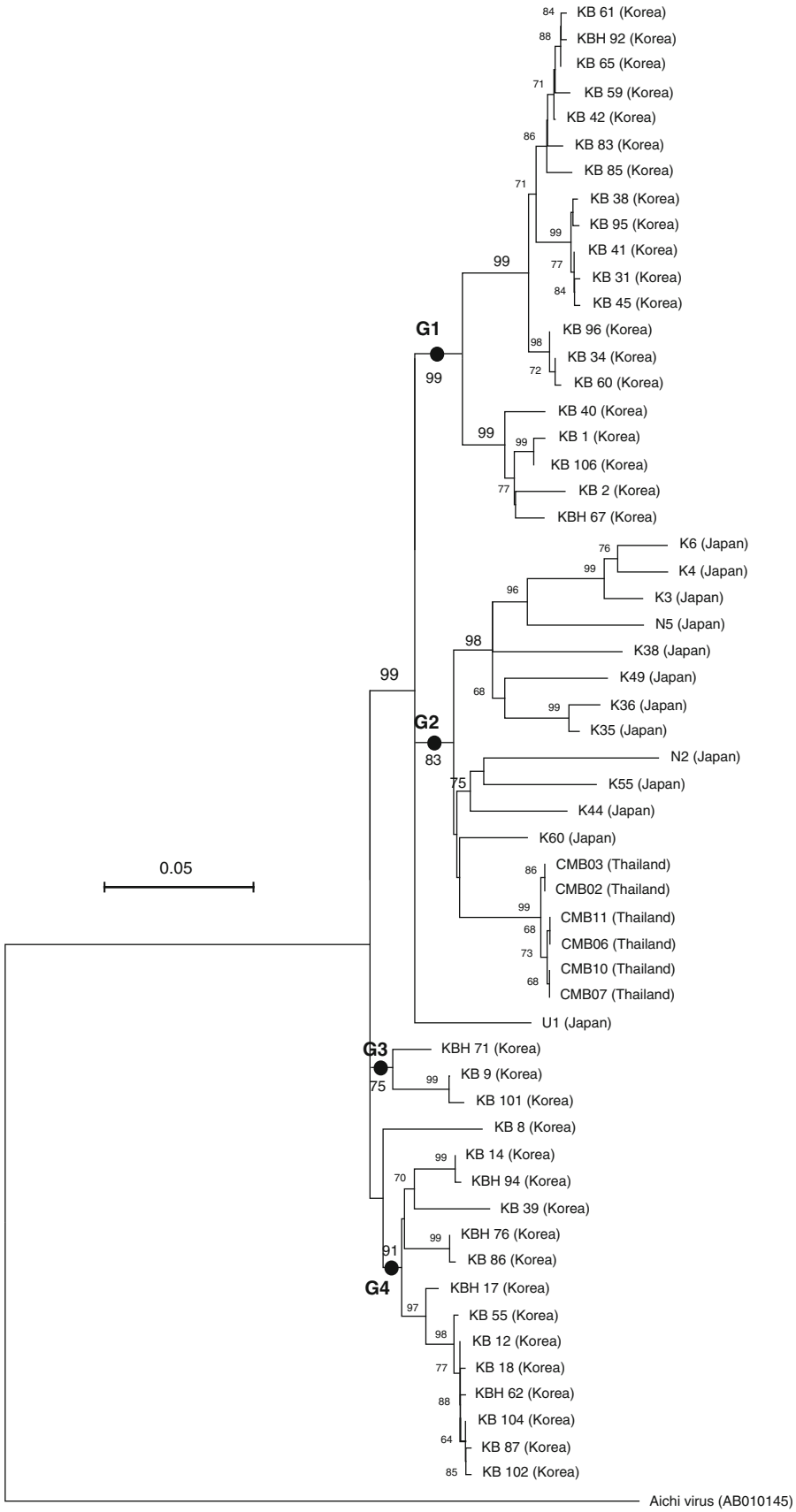

Fig. 1 Neighbor-joining tree for nucleotide sequences of bovine kobuvirus strains. Partial nucleotide sequences (590 bp in length) from the 3D genes of 56 global bovine kobuvirus (37 Korean, 13 Japanese, and 6 Thailand), along with the Aichi virus as an outgroup, are shown. Bootstrap percentages are shown above those nodes that are supported in at least 60% of the 1,000 replicates. *Scale bar* indicates nucleotide substitutions per site

of infected cattle, this study clearly showed a predominance of infection in calves under the age of 1 month ($n = 25$), a result similar to that of a previous study [5]. Kobuvirus prevalence by geographic region was 45.2% (19/42) in Chungnam, 30% (3/10) in Gyongbuk, 28.1% (9/32) in Gyonggi, 27.3% (3/11) in Chungbuk, 25% (2/8) in Gangwon, and 25% (1/4) in Gyongnam. In spite of these seemingly substantial differences in prevalence, the low numbers of samples did not provide sufficient statistical power to allow any conclusions regarding geographic predilection. The geographic differences can therefore be considered tenuous at this point, requiring further study with larger sample sizes. The clinical significance of any such differences remains unclear.

The combined infection involving bovine kobuvirus and other viruses was observed in nine cattles: BRV ($n = 3$), BCV ($n = 1$), BVDV ($n = 1$), and BCV + BVDV ($n = 4$). However, it is unclear whether the other viruses are directly associated with the kobuvirus infection. Neighbor-joining analysis revealed that partial nucleotide sequences (590 bp in length) of the 3D genes of 56 bovine kobuvirus (37 from Korea, 13 from Japan, and 6 from Thailand), along with that of the Aichi virus (as the outgroup), fell into four main lineages (G1, G2, G3, and G4). With the exception of the U1 and KB8 strains, all of the sequences fell into one of these four lineages (Fig. 1). The four lineages were supported by high bootstrap values (75–99%) at the node of each branch. Interestingly, the 36 Korean kobuvirus strains formed three lineages (G1, G3, and G4), while the 12 Japanese and six Thailand strains all fell within the G2 lineage (Fig. 1). A future analysis using a larger number of strains may be required to confirm that the U1 and KB8 strains represent the first recognized strains of an additional cluster or two additional clusters.

In conclusion, the findings of this study demonstrate the existence of four phylogenetic lineages of bovine kobuvirus. Korean kobuvirus strains are found in three of the four lineages, with Japanese and Thailand strains being clustered together in the other lineage.

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