

Study on Molecular Evolution of Orbiviruses

(オルビウイルス流行株の変異に関する分子遺伝学的研究)

Seiichi OHASHI

Contents

Preface	1
Chapter I Identification and PCR-Restriction Fragment Length Polymorphism Analysis of a Variant of the Ibaraki Virus from Naturally Infected Cattle and Aborted Fetuses in Japan.	
1. SUMMARY	7
2. INTRODUCTION	8
3. MATERIALS AND METHODS	8
4. RESULTS	
4.1. Virus isolation	11
4.2. Neutralization test	12
4.3. Genomic dsRNA profiles of viruses	12
4.4. RT-PCR	12
4.5. RFLP analysis of PCR products	13
5. DISCUSSION	13
Chapter II Analysis of Intratypic Variation Evident in an Ibaraki Virus Strain and Its Epizootic Hemorrhagic Disease Virus Serogroup.	
1. SUMMARY	17
2. INTRODUCTION	18
3. MATERIALS AND METHODS	18
4. RESULTS	

4.1. Sequence determination and analysis	20
4.2. Phylogenetic analysis	21
5. DISCUSSION.....	22
Chapter III Evidence of an Antigenic Shift among Palyam Serogroup Orbiviruses.	
1. SUMMARY.....	27
2. INTRODUCTION.....	28
3. MATERIALS AND METHODS.....	29
4. RESULTS	
4.1. Partial sequence and phylogenetic analysis of segment 7.....	32
4.2. Sequence and phylogenetic analysis of the full length of Segment 2.....	32
4.3. Cross-neutralization test.....	34
5. DISCUSSION.....	34
CONCLUSION.....	38
ACKNOWLEDGEMENTS.....	41
REFERENCES.....	42
FIGURES and TABLES.....	50

Preface

The orbivirus is an arthropod-borne virus. These viruses are transmitted between vertebrate hosts by blood-sucking arthropods such as *Culicoides* biting midges and replicate both in vertebrates and arthropods. The viruses are transmitted transboundary by blood-sucking arthropods. Therefore, once the virus invades a naive area, the viruses are spread extensively and the diseases occur epizootically. The diseases caused by orbiviruses are distributed over both temperate and tropical regions of the world, including Africa, America, Australia, and Asia including Japan.

Orbiviruses are responsible for a number of economically important diseases in domestic and wild animals, such as *Bluetongue virus* (BTV) and *African horse sickness virus* (AHSV), both of which are regarded as the most important animal diseases affecting the international trade of animals and their products, and *Epizootic hemorrhagic disease virus* (EHDV) and *Palyam virus* (PALV), which cause from fatal to subclinical disease in ruminants. BTV and EHDV affect ruminant species, and AHSV affects equine species. There are a variety of symptoms in vertebrate hosts in response to orbivirus infection, ranging from inapparent or mild febrile response to more severe infection.

There are 24 serotypes in the BTV serogroup. BTV strains have been distributed in Australia (serotypes 1, 3, 9, 15, 16, 20, 21, and 23), the United States (serotypes 2, 10, 11, 13, and 17), China (serotypes 1, 2, 3, 4, 7, 9, 10, 12, 17, 21, and 23), Southeast Asia (serotypes 1, 2, 3, 4, 7, 9, 10, 12, 17, 21, and 23), and Africa (all serotypes other than 18, 21, and 23). Although infection of cattle with BTV is usually asymptomatic, a sheep may have a high fever and distinctive lesions in the mouth, including a tongue that becomes severely affected and turns dark blue. This type of BTV infections occurs as a clinical disease in several countries in the Mediterranean region, South Africa, the Middle East, India, China, and the United States. Curiously, there has been no evidence of any clinical disease associated with BTV infection of any livestock species in Australia.

EHDV has primarily been associated with hemorrhagic disease in white-tailed deer in North America (3). There is also evidence of infection of domestic animals (56). The EHDV serogroup consists of 8 serotypes. There are two serotypes (serotype 1 and 2) of EHDV in North America. Serotypes 3 and 4 were isolated in Nigeria. Six serotypes of EHDV (serotypes 1, 2, 5, 6, 7, and 8) have been recovered from blood of cattle and/or *Culicoides brevitarsis* in Australia. Although infections with EHDV have been confirmed in cattle, buffalo, and deer, clinical diseases in these animals caused by EHDV have not been observed in Australia (Fig. 1).

African horsesickness is one of the most lethal diseases, with mortality rates ranging from 40-90%. African horse sickness has been observed in restricted areas of the Middle East and Africa. AHSV can cause four clinical types of the disease in horses. The febrile form is relatively mild, and infected animals are recovered. The pulmonary form is more severe and is nearly fatal. The third type is cardiac and is characterized by fever and edema of the head and neck. The cardiac form of African horse sickness is common in horses in enzootic areas. The fourth type is a mixed form between the pulmonary and cardiac forms of the diseases and is characterized by high fever, but 100% recovery (49).

The Orbiviruses, members of the family *Reoviridae*, were initially divided into 19 serogroups on the basis of cross-reaction of isolates in a complement fixation test, agar gel immunodiffusion, and a fluorescent antibody test (34). There are a number of serotypes within the serogroup that can be classified by a serum neutralization test.

There is evidence of nucleotide and amino acid sequence variation depending on the geographical origin of the virus isolate, regardless of the virus serotype. These variations may reflect the accumulation of mutations and reassortment and coevolution of common gene pools at different locations such as Asia, Australia, Africa, and North America. Gould and Pritchard have suggested that the “topotype” should be applied to each distinct group (18). However, orbiviruses circulating in Japan and neighboring countries have not been investigated genetically and

antigenically. It is necessary for understanding ecology of orbivirus circulating East Asia to elucidate genetic and antigenic relationship between orbiviruses.

Orbiviruses are nonenveloped, icosahedral particles 60-80 nm in diameter with double-layered capsids and a genome consisting of 10 double-stranded RNA (dsRNA) segments. The dsRNA segments are identified as segments 1-10, ranging in length from 3957 bp to 822 bp in BTV. These segments code for seven structural proteins (segment 1- VP1, segment 2- VP2, segment 3- VP3, segment 4- VP4, segment 6- VP5, segment 7- VP7, and segment 9- VP6) and four nonstructural proteins (segment 5- NS1, segment 8- NS2, and segments 10- NS3 and NS3A) (26, 57). The RNA segments can be separated by agarose gel electrophoresis into patterns distinctive for each serogroup.

Each dsRNA segments of orbivirus codes for one major protein. The outer capsid proteins of the virion contain two major proteins, VP2 and VP5. The core particle contains two major (VP3 and VP7) and three minor proteins (VP1, VP4, and VP6). Four nonstructural proteins are also synthesized in infected cells, together with the seven structural proteins.

The VP2 is the determinant of serotype, inducing neutralizing antibody and involving the attachment site of the virus to vertebrate cells. When VP2 is removed from the virion, the infectivity of the virus on vertebrate cells is significantly reduced (21, 24). The second outer capsid protein, VP5, may also contribute in the induction of neutralizing antibody in BTV and EHDV. VP3 and VP7 are major structural proteins of the inner capsid. VP3 contains group-specific antigenic determinants. The VP7 also contains group-specific antigen and is involved with binding to insect cells (61).

The three minor core proteins, VP1, VP4, and VP6, are coded for by RNA segments 1, 4, and 9, respectively. The VP1 protein is the virion-associated RNA-dependent RNA polymerase. VP4 possesses various enzymatic activities. The protein has guanylyl transferase activity and also methyltransferase activity for methylation of capped orbivirus RNA. The VP6 protein has been

demonstrated to have high binding affinity for both ssRNA and dsRNA. In addition, RNA-dependent ATPase and helicase activities have been identified (46, 51).

The four nonstructural proteins, NS1, NS2, NS3, and NS3A have been identified in infected cells. NS1 is encoded by dsRNA segment 6 and is synthesized in large quantities, forming tubular structures in infected cells. The tubules are predicted to be involved in virus assembly. NS2 is located in the cytoplasm of infected cells as viral inclusion bodies. NS2 is encoded by dsRNA segment 8 and is the only virus-specific phosphoprotein. The protein possesses ssRNA-binding activity. Both NS3 and NS3A, which are related to each other, are encoded by dsRNA segment 10. NS3A is synthesized from the second in-frame translation initiation codon. Both proteins are integral membrane glycoproteins and are involved in the release of virus particles from infected cells.

In orbivirus, the serological features of virus isolates are determined by each protein encoded by RNA segments 2, 3, 6, and 7, respectively, while RNA segment 3 sequences determine the topotype of the BTV isolates. Segment 2 exhibits a higher degree of RNA sequence variation than other genome segments, thus reflecting its involvement in determination of the virus serotype. If reassortment in segment 2 has occurred, it may influence the serotype of progeny viruses and may play a role in the generation and evolution of new virus serotypes.

Analyses of RNA sequence variation between orbivirus isolates circulating Japan will improve our understanding of the regional distribution and the evolutionary relationships of the different virus isolates and will help to confirm any antigenic alterations.

In Japan, several orbivirus serogroups are present. Ibaraki virus (IBAV), a member of the EHDV serogroup, and Chuzan virus (CHUV), a member of the PALV serogroup, were recovered and are known to cause diseases in cattle in Japan. IBAV is transmitted by *Culicoides* biting midges and causes Ibaraki disease, which is characterized by fever, anorexia, and a deglutitive disorder in cattle (41, 42). Since the first recognition of the disease in Japan in 1959, epizootic

occurrences of the disease have been reported in Korea and Taiwan as well as Japan (1, 31) and antibodies to the virus have been found in bovine sera in Australia and Indonesia (8, 13, 35). There are eight different serotypes in the EHDV serogroup (14). Although IBAV is distinguishable from the eight other serotypes of EHDV within the serogroup, a partial cross-neutralization between IBAV and the Alberta strain of EHDV-2 has been found (4, 12, 52). CHUV was isolated from the biting midge *Culicoides oxystoma* and from sentinel calves in 1984 and 1985 in Japan (30, 36). The virus was subsequently implicated in an epizootic occurrence of congenital abnormalities with hydranencephaly-cerebellar hypoplasia syndrome in calves observed in the Kyushu district from autumn 1985 through spring 1986.

Therefore, the purpose of this study is to clarify the genetic and antigenic variations of orbiviruses circulating within East Asia, including Japan, and also to investigate the effects on the pathogenicity by reassortment. Genetic and antigenic analysis of orbiviruses that have been isolated in Japan will contribute to molecular diagnosis for orbivirus infection and more effective vaccine development.

The present thesis consists of 3 chapters. In the first chapter, the suspected causal agent was identified and genetically compared to previous isolates of IBAV and EHDV by PCR-restriction fragment length polymorphism analysis. In the second chapter, the sequencing and comparison of four RNA segments of IBAV, including those of the new strain, are described along with the sequence data of related viruses in order to demonstrate the genetic heterogeneity within the EHDV serogroup as well as the consequences of virus evolution. In the last chapter, the Japanese isolates of PALV serogroup viruses are analyzed so as to determine the genome sequences of segments 2 and 7 and to compare them phylogenetically with Australian and African isolates of the PALV.

Chapter I

Identification and PCR-Restriction Fragment Length Polymorphism Analysis of a Variant of the Ibaraki Virus from Naturally Infected Cattle and Aborted Fetuses in Japan

SUMMARY

One hundred fourteen field isolates of IBAV, a member of the EHDV, were isolated from blood samples of affected and apparently healthy cattle and *Culicoides* biting midges and from blood samples of dams and internal organs of aborted fetuses during an outbreak of Ibaraki disease in the southern part of Japan in 1997. In this outbreak, 242 cattle showed typical symptoms of the disease, and several hundred dams had miscarriages or stillbirths. The viruses that induced typical Ibaraki disease and reproductive problems among cattle were identical and were antigenically closely related to but distinct from previous isolates of IBAV and EHDV-2. The virus was considered to be a putative agent of this outbreak. Reverse transcription (RT)-PCR based on segment 3 of the RNA genome of EHDV-2 and restriction fragment length polymorphism (RFLP) analysis of the PCR products were conducted to compare the genomes of the viruses. The results suggested that the virus isolated in 1997 was a variant of IBAV and might be exotic.

INTRODUCTION

Ibaraki disease is an arthropod-borne viral disease of cattle characterized by fever, anorexia, and deglutitive disorder (41, 42). Since the first recognition of the disease in Japan in 1959, epizootic occurrences of the disease have been reported in Japan, Korea, and Taiwan (1, 31). The causative agent of Ibaraki disease is IBAV, which belongs to the EHDV serogroup in the genus *Orbivirus* in the family *Reoviridae*. Eight different serotypes of EHDV are known to exist worldwide (14). IBAV has been demonstrated to be closely related serologically but to distinct from the Alberta strain of EHDV-2 (4, 52).

The virion contains 10 double-stranded (ds) genome segments. Each of the segments encodes various structural and nonstructural viral proteins (22, 23, 33). One of these, segment 3 of the RNA genome, encodes a serogroup-specific antigen, VP3. Recently, researchers demonstrated that reverse transcription (RT)-PCR with primers based on the sequence of segment 3 was a useful tool for the detection and differentiation of the EHDV serogroup (20, 32).

Ibaraki disease occurred on an epidemic scale in the late summer to autumn of 1982 and 1987 in the western part of Japan. After the last outbreak in 1987, the next epidemic of the disease occurred from August to November 1997 in the same area. In the latter outbreak, numerous abortions and stillbirths were reported among cattle, in addition to the typical symptoms of Ibaraki disease. The viruses were isolated from the blood of affected and apparently healthy animals and from aborted fetuses and *Culicoides* biting midges. This chapter describes the identification of the suspected causal agent and the genetic comparison with previous isolates of IBAV and EHDV by PCR-RFLP analysis.

MATERIALS AND METHODS

Viruses and cells. The Ibaraki-2 (42) and Y87061 strains of IBAV were isolated from the blood of infected cows in 1959 and 1987, respectively. The following EHDV strains were used in this

experiment: New Jersey (serotype 1), Alberta (serotype 2), CSIRO439 (serotype 2), CSIRO157 (serotype 5), CSIRO753 (serotype 6), CSIRO775 (serotype 7), and DPP 59 (serotype 8). All viruses were propagated in hamster lung (HmLu-1) cells and baby hamster kidney (BHK-21) cells. The cells were grown in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 0.15% sodium bicarbonate, 2 mM L-glutamine, and 10% calf serum. The viruses were inoculated onto a cell monolayer that had been washed three times with Earle's solution and were cultured with serum-free MEM. Infectious culture fluid was harvested when the cells showed a complete cytopathic effect (CPE).

Virus isolation. The heparinized blood samples were collected from cattle with typical symptoms of Ibaraki disease and from cattle raised in the same cowshed with the affected animals. Blood samples were also taken from dams which had a miscarriage or a stillbirth. These blood samples were separated into plasma and erythrocytes, and the erythrocytes were washed three times with phosphate-buffered saline to eliminate the antibodies. The internal organs of the fetuses, the placenta, and *Culicoides* biting midges were homogenized with MEM. All samples were stored at -80°C until use. The cells grown in test tubes were washed three times with Earle's solution before inoculation with samples. After inoculation with 0.1 ml of the samples, the cells were incubated with 0.4 ml of MEM supplemented with antibiotics at 37°C for 7 days. The cultures were passaged in the same manner until a CPE was observed.

Production of hyperimmune serum. Hyperimmune sera against viruses were raised in rabbits. Fluid from cultures of the virus in BHK-21 cells was centrifuged at $5,600 \times g$ for 20 min at 4°C and was concentrated by precipitation with 50% saturated ammonium sulfate. Purified virus was obtained at the interface of the discontinuous gradient of 20% and 50% (wt/vol) sucrose in phosphate-buffered saline after centrifugation at $100,700 \times g$ for 2 h at 4°C (SW 28.1 rotor; Beckman Coulter Inc., Fullerton, Calif.). The rabbits were immunized once intradermally with a

mixture of purified virus and Freund's complete adjuvant (Dia-iatron Co., Ltd., Tokyo, Japan) and, 3 weeks later, subcutaneously with a mixture of purified virus and Freund's incomplete adjuvant (Dia-iatron Co., Ltd.). The serum was collected 10 days after the booster immunization.

Serum neutralization test. Antiserum was serially diluted twofold with serum-free MEM in a flat-bottom 96-well microplate (Sumitomo Bakelite Co., Tokyo, Japan). One hundred 50% tissue culture infective doses of virus were added to each dilution. The mixtures were incubated at 37°C for 1 h, and then HmLu-1 cells suspended in serum-free medium (GIT; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to each well. After incubation at 37°C for 7 days in a humidified 5% CO₂ atmosphere, the antibody titers were expressed as a reciprocal of the highest dilution of sera that completely inhibited the CPE.

Preparation of viral dsRNA. The viral dsRNA was extracted from infected BHK-21 cells by the method described by Siaz-Ruiz and Kaper (48). Briefly, infected BHK-21 cells were homogenized in TE buffer (2 mM Tris-HCl, 1 mM EDTA [pH 8.0]). After centrifugation, the supernatant was disrupted in 1% sodium dodecyl sulfate–0.4 M NaCl. The nucleic acid was precipitated by phenol extraction and ethanol precipitation. The pellet was resuspended in 1 mM EDTA solution (pH 5.0), and then an equal volume of 4 M LiCl solution was added and the mixture was kept at 4°C for 8 h. After removal of the precipitant by centrifugation, an equal volume of 8 M LiCl solution was added to precipitate the viral dsRNA, and the mixture was kept at 4°C for 8 h. The final pellet was reconstituted in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The viral dsRNA was separated on a 0.9% agarose gel (FMC Bioproducts, Rockland, Maine) for 60 min at 100 V. The gel was stained with ethidium bromide and was visualized under UV light and photographed.

RNA extraction and RT-PCR. Viral RNA was extracted from virus culture fluid with a High Pure Viral RNA kit in accordance with the manufacturer's instruction (Roche Diagnostics, Indianapolis, Ind.). The primers (L3-1: 5'-CCCAGATGTTCAATAGCGAACCTAATC-3' and

L3-2: 5'-TAACATTTTCGTTATAGCAATAGTAGTT-3') were synthesized on the basis of the sequence of segment 3 of EHDV described elsewhere (32). RT-PCR was performed with a TaKaRa RNA PCR kit (with RTase from avian myeloblastosis virus), version 2.1 (TaKaRa Shuzo Co., Ltd., Shiga, Japan), with some modifications. Briefly, the cDNA was synthesized at 42°C for 30 min after preincubation with both primers at 94°C for 4 min and then on ice. For PCR amplification, the PCR mixture (5 mM MgCl₂, 1× PCR buffer, 2.5 U of Taq polymerase) was added to the RT reaction mixture. PCR was carried out with 28 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 90 s. The PCR products were separated on a 1.5% agarose gel at 100 V for 40 min. After staining with ethidium bromide, the gel was visualized under UV light and photographed.

RFLP analysis of PCR products. The complete nucleotide sequence of the RNA segment 3 of an Australian isolate of EHDV-2 and the partial nucleotide sequence of IBAV have been reported elsewhere (18). The predicted PCR products of EHDV-2 and IBAV have been shown to be cut at five and at least two sites, respectively, with restriction enzyme *Sau3A* I, and those of IBAV has been shown to be cut at at least one site with restriction enzyme *Hae* III. The PCR products were digested with either *Hae* III or *Sau3A* I (TaKaRa Shuzo Co., Ltd.), followed by ethanol precipitation. All reactions were performed at 37°C for 60 min. Restriction fragments were separated on a 2.0% agarose gel, and the gel was observed as described above.

RESULTS

Virus isolation. Ibaraki disease occurred from August through November 1997 in nine prefectures in the western part of Japan. During that outbreak, 242 cattle showed clinical symptoms and were diagnosed with Ibaraki disease on the basis of serological examination to confirm the seroconversion to IBAV. During the same period, several hundred cases of abortions and stillbirths occurred among cattle in the same prefectures. A total of 114 virus isolates were

obtained from blood samples from both affected and apparently healthy cattle and from the samples of the fetuses and *Culicoides* biting midges. Fifty-three, 3, and 11 isolates were isolated from erythrocytes, plasma, and whole blood, respectively, of cattle exhibiting typical Ibaraki disease symptoms and cattle raised in the same cowshed with affected animals. These 67 isolates are referred to as Ibaraki disease relatives. Thirty-five and two isolates were from the aborted fetuses and placentas, respectively. Seven isolates were obtained from the blood of dams that had a miscarriage or stillbirth and from the blood of cattle raised in the same cowshed with the affected animals. These 44 isolates are referred to as abortion or stillbirth relatives. Three virus isolates were obtained from *Culicoides* biting midges caught during the epidemic period of the disease.

Neutralization test. An isolate, designated KS(H-22)P/97, was chosen from among the isolates of Ibaraki disease relatives, and its serological relationship to IBAV and EHDV was investigated via the cross-neutralization test. As shown in Table 1-1, antisera to the Ibaraki-2 and Y87061 strains of IBAV equally neutralized the homologous strain at titers up to 64 to 128 as well as the CSIRO439 strain of EHDV. The Alberta strain of EHDV was neutralized by antisera to Ibaraki-2 and Y87061 at titers up to 8 and 16, respectively. KS(H-22)P/97 was neutralized by these sera up to a titer of 8. On the other hand, antiserum to KS(H-22)P/97 neutralized the homologous strain at a titer of 64, whereas the neutralization titers of the serum against other strains of IBAV and EHDV were less than 16.

Genomic dsRNA profiles of viruses. The genomic dsRNA profile of KS(H-22)P/97 was determined by agarose gel electrophoresis and was compared with those of strains Ibaraki-2 and Y87061 of IBAV and the CSIRO439 strain of EHDV (Fig. 1-1). The migration patterns of the genome segments of strain KS(H-22)P/97 were identical to those of the other three strains of the virus. Segments 7 and 8 of the viruses seemed to migrate together.

RT-PCR. To confirm the specificity of RT-PCR with the synthesized primer pair, PCR was done with cDNA transcribed from dsRNA extracted from IBAV and a different serotype of EHDV.

The specific PCR product, with an expected size of 659 bp, was amplified from the Australian EHDV isolates, i.e., CSIRO439, CSIRO157, CSIRO753, CSIRO775, and DPP 59, as well as the Ibaraki-2 and Y87061 strains of IBAV (Fig. 1-2A, Fig. 1-2B). No specific band was amplified for the North American EHDV isolates, the New Jersey and Alberta strains (Fig. 1-2A). The specific band was amplified from Ibaraki disease relatives, abortion or stillbirth relatives, and isolates from *Culicoides* biting midges in 1997. Figure 1-2B shows the results of RT-PCR for representative isolates from different prefectures, Hyogo (HG), Okayama (OY), Fukuoka (FO), Saga (SG), Nagasaki (NS), Kumamoto (KM), Oita (OI), Miyazaki (MZ), Kagoshima (KS), and Okinawa (ON).

RFLP analysis of PCR products. The identities of the specific PCR products were analyzed by RFLP analysis. The PCR products were digested with either the *Hae* III or the *Sau*3A I restriction enzyme. Analysis of the RFLP patterns obtained with *Hae* III grouped the viruses into two types of Australian EHDV and IBAV strains. All 114 isolates recovered in 1997 had the same RFLP pattern as those of IBAV (Fig. 1-3A). The RFLP patterns obtained with *Sau*3A I were rather complicated (Fig. 1-4B). Three Australian EHDV isolates, CSIRO439, CSIRO157, and CSIRO775, had the same RFLP pattern as the Ibaraki-2 strain of IBAV. Australian EHDV strains CSIRO753 and DPP 59 and IBAV strain Y87061 each had different RFLP patterns. However, all 114 isolates recovered in 1997 had identical RFLP patterns, and the patterns were distinct from those of EHDV and formerly recovered IBAV isolates (Fig. 1-3B).

DISCUSSION

During the epidemic of Ibaraki disease in 1997, affected cattle showed typical symptoms of the disease, i.e., fever, anorexia, and disturbance of deglutition. Sixty of the animals died of aspiration pneumonia, dehydration, and emaciation resulting from difficulty with swallowing. In such animals, degeneration of striated muscular tissue was observed in the esophagus, larynx, pharynx,

tongue, and skeletal muscles. These clinical and pathological findings are identical to those that occur with Ibaraki disease (41). Although subclinical or inapparent infection with IBV has frequently occurred, abortion and stillbirth of cows had not been reported in previous outbreaks. During the outbreak in 1997, numerous cases of abortions and stillbirths among cattle were observed together with Ibaraki disease. A total 114 virus isolates were obtained from the blood of affected animals and cattle raised in the area where the epidemic occurred and from fetuses and *Culicoides* biting midges that were suspected as being the vectors. The results of RFLP analysis with *Hae* III and *Sau*3A I with these viruses revealed that all of the isolates were identical and had the same origin. Furthermore, the virus seemed to cause not only typical symptoms of Ibaraki disease but also abortions and stillbirths in cows.

The results of a neutralization test indicated that strain KS (H-22)P/97 was closely related to IBV and EHDV-2. However, the isolate was distinct from strains of both virus groups. Strain CSIRO439 was most closely related to the prototype Ibaraki-2 and Y87061 strains of IBV, as described previously (5), while strain KS(H-22)P/97 was distinct from the Ibaraki-2 and Y87061 strains of IBV and the CSIRO439 strain of EHDV-2. The antigenic relationship between KS(H-22)P/97 and the North American EHDV-2 strains is not clear. The genomic dsRNA profile revealed that strain KS(H-22)P/97 belongs to the same genotype that includes the Ibaraki-2, Y87061, and CSIRO439 strains.

The sequence of RNA segment 3 of EHDV has been demonstrated to be highly conserved, with more than 90% homology among cognate genes of the same EHDV topotype (5, 18, 59). The primer pair based on the sequence of the RNA segment 3 of EHDV allowed successive detection of Australian EHDV and IBV strains as well as isolates recovered in 1997. McColl and Gould (32) revealed that the specific PCR product of IBV was not obtained at the high-stringency annealing temperature at 65°C but that it was obtained when the temperature was reduced to 37°C. In this experiment, the specific products were obtained from both EHDV and IBV at an

annealing temperature of 60°C. The serogroup-specific primer used in this study, however, did not amplify the genes of the North American EHDV strains. Although the nucleotide sequences of segments 3 of the Australian EHDV and North American EHDV strains had 79% homologies (18, 59), the nucleotide sequences of the North American EHDV strains were substituted at the 3' end of the primer annealing site. The failure of specific amplification in North American EHDV strains might be caused by this substitution. The RT-PCR could not directly distinguish IBAV from Australian EHDV strains, contrary to the results described by McColl and Gould (32).

RFLP analysis of PCR products allowed grouping of the viruses. Analysis of the RFLP pattern obtained by digestion with *Hae* III grouped viruses into distinct topotypes of Australian EHDV and IBAV (Japanese EHDV) strains. The isolates recovered in 1997 were grouped into IBAV. Although the RFLP pattern obtained by digestion with *Sau*3A I indicated that the genomic variation existed in a topotype among Australian EHDV and IBAV strains, further information including determination of the nucleotide sequence would be needed to reveal the phylogenic association. The RFLP pattern obtained by digestion with *Sau*3A I suggested that the isolates recovered in 1997 were unique strains of IBAV. The serological surveillance data indicated that many arboviruses, including EHDV and IBAV, are present in Eastern and Southeastern Asia (8, 35). In Japan, import controls on animals and preventive efforts with vaccines have been used. The live attenuated vaccine derived from the Ibaraki-2 strain has been demonstrated to be effective and safe. National surveillance and intensive monitoring of yearlings as sentinel cattle have been in place for a number of years, and until 1997, Ibaraki disease had not been observed and no animals in the sentinel groups had seroconverted since 1987. In addition to this history, the fact that the outbreak of Ibaraki disease and reproductive problems had occurred in southern part of Japan in 1997 suggests that the virus was probably introduced into Japan by infected *Culicoides* biting midges carried on the wind from places where climate conditions were suitable.

Chapter II

Analysis of Intratypic Variation Evident in an Ibaraki Virus Strain and Its Epizootic Hemorrhagic Disease Virus Serogroup

SUMMARY

A new strain of IBAV was isolated from cattle showing atypical symptoms of Ibaraki disease. The isolate was genetically characterized, and the genetic diversity and evolution of the capsid proteins of viruses in the EHDV serogroup were investigated. The nucleotide sequences of the isolate's viral RNA segments 2, 3, 6, and 7, which encode the viral structural proteins VP2, VP3, VP5, and VP7, respectively, were determined and were then compared against those of the existing strains of IBAV and EHDV, to which IBAV belongs serologically. The nucleotide sequences of segments 3 and 7 were conserved within the EHDV serogroup, particularly well among the strains of IBAV and Australian EHDV. The similarity of the sequence of segment 6 of the isolate to sequences of corresponding segments of the other strains of IBAV and EHDV was found to be about 93%. The similarity of segment 2 of the isolate to those of the other strains of IBAV and EHDV was less than 70%. Phylogenetic analysis based on the deduced amino acid sequences of segments 3 and 7 revealed that the viruses differed according to their geographical distributions. However, the new isolate of IBAV was categorized as having a distinct lineage in the phylogenetic tree of VP2. These results suggest that the isolate was modified by a reassortment of segment 2 and that it exhibits unique genetic and antigenic characteristics.

INTRODUCTION

Ibaraki disease was initially reported in Japan in 1959, and since then there have been epidemics of it in east Asia (1, 31) and antibodies to the virus have been found in bovine sera in Australia and Indonesia (8, 13, 35).

The IBAV genome is composed of 10 dsRNA segments in a bilayered capsid. Segments 3 and 7 encode the inner capsid proteins VP3 and VP7, respectively. These capsid proteins play the role of serogroup-specific antigens. Segments 2 and 6 encode the outer layer proteins VP2 and VP5, respectively, of which VP2 is a major neutralizing antigen and serotype-specific determinant (23). The 10 genomic segments have the potential to be exchanged within the serogroup (reassortment). In another orbivirus, BTV, reassortment events in tissue culture, in the vector, and in nature have been described (9, 11, 45). The molecular characterization of IBAV would be valuable for investigating the viral evolution and epidemiology of the disease.

The results from chapter 1 demonstrated that new virus was distinguished from the existing strains of IBAV by a cross-neutralization test and by PCR-RFLP analysis. In this chapter, four RNA segments of IBAV, including the new strain, were sequenced and compared with the published sequence data in order to demonstrate the genetic heterogeneity within the EHDV serogroup, along with the consequences of virus evolution.

MATERIALS AND METHODS

Viruses and cells. All viruses using in this study were propagated in HmLu-1 cells and BHK-21 cells. The Ibaraki-2, Y87061, and KSB-14/E/97 IBAV strains, isolated in 1959, 1987, and 1997, respectively, and the CSIRO439 strain of Australian EHDV-2 were propagated in BHK-21 cells. The cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co.) supplemented with 0.295% tryptose phosphate broth (Difco Laboratories), 0.15% sodium bicarbonate, 2 mM L-glutamine, and 5% calf serum.

Extraction of viral dsRNAs. Viral dsRNAs were extracted from the infected BHK-21 cells as described previously (48), and the individual RNA segments were separated on a 0.8% agarose gel. The separated viral dsRNAs were excised from the agarose gel and purified with the RNaid kit (Bio 101, La Jolla, Calif.) in accordance with the manufacturer's instructions.

Genomic amplification and cloning. The primers corresponding to the 5' and 3' termini of segments 2, 3, 6, and 7 were synthesized on the basis of the sequences of the No.2 strain of IBAV (Table 2-1) (25, 39, 40). RT-PCR was performed with the Titan One Tube RT-PCR kit (Roche Diagnostics) containing a proofreading polymerase. The RT-PCR mixture consisted of 0.2 mM deoxynucleoside triphosphate, 5 mM dithiothreitol, 1 × RT-PCR buffer, 1.5 mM MgCl₂, and an enzyme mixture in a total reaction volume of 50 μl. The purified viral RNA segment and both strand primers were incubated at 94°C for 5 min and quenched on ice prior to the addition of the reaction mixture. The RT was conducted at 48°C for 60 min. This was followed by an initial 3-min denaturation at 95°C and 10 subsequent cycles of denaturation at 95°C for 30 s, annealing at 55 to 63°C (depending on the primer pair) for 30 s, and elongation at 68°C for 2 min, and then by 25 cycles of denaturation at 95°C for 30 s, annealing at 55 to 63°C for 30 s, and elongation at 68°C for 2 min at first, though with each cycle the elongation time was incremented by 5 s. The purified PCR products were ligated into pGEM-T Easy Vector systems (Promega, Madison, Wis.). The recombinant plasmids were transfected into competent *Escherichia coli* JM 109 cells. The positive clones were confirmed by PCR using M13 forward and reverse primers. A plasmid containing the viral genome was obtained with a commercial plasmid extraction kit (Promega) according to the manufacturer's protocol.

Sequencing. The sequencing primers are shown in Table 2-1. Three individual clones containing the viral genome were sequenced in both directions by using a model 4200 automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) with the Thermo Sequenase cycle sequencing kit (U.S. Biochemicals, Cleveland, Ohio).

Genetic analysis. The nucleotide sequences obtained in this study were edited with the GENETYX software. The multiple alignments of the deduced amino acid sequences encoded by the open reading frames (ORF) were done with the CLUSTAL W program (55). The aligned sequences were used to construct the phylogenetic trees by the neighbor-joining method (47), and the distances were corrected by using Kimura's two-parameter method (28). The reliability of the branching orders was estimated by bootstrapping (1,000 samples). The phylogenetic trees were drawn with TreeView software (43).

Sequence data. The sequence data determined in this study and previously reported nucleotide sequences of IBAV and Australian and North American EHDV segments 2, 3, 6, and 7 were used in the genetic analysis. The sequence data were taken from the nucleotide database (the accession numbers are shown in the legends for Fig. 2-1 and 2-2).

Nucleotide sequence accession numbers. The IBAV and Australian EHDV-2 genome sequences reported in this study have been deposited in the DDBJ, EMBL, and GenBank databases and given the following accession numbers: AB078620 through AB078633.

RESULTS

Sequence determination and analysis. cDNA clones were generated for segments 2, 3, 6, and 7 of the strains Ibaraki-2, Y87061, and KSB-14/E/97 of IBAV and for segments 2 and 6 of the strain CSIRO439 of EHDV-2. To reveal the phylogenetic relationships within the EHDV serogroup, these clones were sequenced, and the sequence data were compared against the published data for strain No.2 of IBAV, strain CSIRO439 of Australian EHDV-2, strains SV 123 and Alberta of North American EHDV-1, and strain SV 124 of North American EHDV-2.

The ORFs of segments 3 and 7 of all the strains of IBAV comprised 2,700 and 1,050 nucleotides, respectively. The variations of segments 3 and 7 within the strains of IBAV and CSIRO439 ranged from 92.8 to 93.7% for segment 3 and from 92.6 to 94.1% for segment 7,

whereas the variations between segments 3 and 7 of IBAV together with CSIRO439 and those of SV 124 of EHDV-2 were both approximately 80% (Table 2-2). The deduced amino acid sequences of VP3 and VP7 were relatively conserved at more than 99% for the IBAV strains, except for the VP7 sequences of strain No.2. The amino acid sequence identities for VP7 between IBAVs and CSIRO439 ranged from 96.0 to 98.9%. The VP3 and VP7 amino acid sequence identities between IBAVs and SV 124 of EHDV-2 ranged from 95.3 to 95.9% for VP3 and from 94.6 to 97.1% for VP7. Similar lower identities between the VP3 and VP7 of CSIRO439 and those of SV 124 were observed.

The ORFs of segments 2 and 6 of Ibaraki-2, Y87061, and KSB-14/E/97 of IBAV and CSIRO439 of EHDV-2 were 2,949 and 1,584 nucleotides in length, respectively. The genomic sequence identities of segment 6 among the strains of IBAV and CSIRO439 of EHDV-2 were between 93.1 and 93.9%. The variations of segment 6 among IBAV strains No.2, Ibaraki-2, and Y87061 were under 2.9%. However, the identities of segment 6 between strain KSB-14/E97 and the other strains of IBAV were 92.2 to 92.6%, indicating a higher degree of genetic change than seen in segments 3 and 7. The deduced amino acid sequences of VP5s of the strains of IBAV and EHDV-2 had relatively high identities, ranging from 97.3 to 99.2%.

A remarkable genomic variation was observed on the nucleotide sequence of segment 2. In contrast with the identities (between 94.4 and 98.5%) of segments 2 of strains No.2, Ibaraki-2, Y87061, and CSIRO439, the identities of segments 2 of KSB-14/E/97 and these strains were less than 70%. Poor identities of the deduced amino acid sequence of KSB-14/E/97 VP2 with those of the other strains of IBAV and EHDV-2 were observed, and the identities had lower values than those between the VP2 sequences of SV 124 and the other strains of IBAV.

Phylogenetic analysis. The phylogenetic trees of VP2, VP3, VP5, and VP7, constructed by using the deduced amino acid sequences encoded by the complete ORFs of segments 2, 3, 6, and 7, were individually compared. In the tree based on the VP3 sequence data (Fig. 2-1) there were two

major clusters. SV 124 of EHDV-2 constituted an individual cluster, and the other cluster was composed of strains of IBAV and CSIRO439 of EHDV-2. Because strain SV 124 was isolated in North America and the other strains were isolated in Asia and Australia, the separation of these clusters seemed to correspond to the geographical distribution of the virus strains. The phylogenetic tree of VP7 resembled that of VP3 in its cluster construction. The clusters in the tree of VP7 were divided into three groups by geographical distribution and might define their geographical gene pool.

The phylogenetic trees of VP5 and VP2 were rather complicated. In the tree of VP5, strain KSB-14/E/97 was separated into a separate cluster (Fig. 2-2). However, strain CSIRO439 was in the same cluster as the existing strains of IBAV. The phylogenetic difference of strain KSB-14/E/97 was demonstrated more clearly in the tree of VP2. Strains No.2, Ibaraki-2, and Y87061 of IBAV were all in the same cluster, which was distinguishable both from the lineage of KSB-14/E/97 and from the lineage of SV 124 (Fig. 2-2).

DISCUSSION

IBAV is one of the most important insect-borne viruses in Japan. Since the first recognition of Ibaraki disease in Japan in 1959, epidemics have occurred in 1960, 1982, and 1987. However, reproductive problems in cows caused by IBAV were not reported until 1997. The serological tests and RFLP analysis of segment 3 of the 1997 isolate revealed that this new isolate belonged to IBAV but it was distinguishable from the IBAVs isolated during past epidemics. This newly identified virus was considered a variant of IBAV in chapter 1. However, genetic characterization of this IBAV had not been performed, and its phylogenetic relationship with other EHDVs, as well as with other strains of IBAV, was unknown. In this study, I determined the sequences of the ORF regions of the four structural protein genes of this new variant virus and compared its nucleotide and deduced amino acid sequences with those of IBAV and EHDV in order to define the

phylogenetic relationship of the virus.

The selected virus strains differed in geographical origin: the IBAV isolate was from Japan, and CSIRO439 strain of EHDV-2 was from Australia, and the other was SV 124 strain of EHDV-2 from North America. I compared sequences of segments 3 and 7, encoding serogroup-specific proteins VP3 and VP7, respectively, from the EHDV serogroup, to one another. The deduced amino acid sequences of the proteins were conserved, having 5% variability, between IBAV and EHDV. In the nucleotide sequences, there is 20% variability between IBAV and North American EHDV, while there is a less than a 7% difference between IBAV and Australian EHDV. The phylogenetic analysis of VP3 showed two distinct clusters between the strains of IBAV and Australian EHDV-2. However, the KSB-14/E/97 strain of IBAV formed a single cluster with the CSIRO439 strain of EHDV-2. The result suggested that IBAV and Australian EHDV share a genetic pool. However, within the EHDV serogroup, phylogenetic analysis of segment 7 produced a more accurate geographical separation than analysis of segment 3, and so North American EHDV might be distinguishable from IBAV and Australian EHDV. Gould et al. demonstrated that a feature of the phylogenetic trees based on VP3 within the EHDV and BTV serogroups could be clearly delineated on the basis of their geographical origins (17, 18, 44), and phylogenetic analyses revealed that the BTV segment 7 genes do not reflect the geographic origins of the virus strains (2, 60). Unlike the phylogenetic analyses of BTV, the phylogenetic analysis of IBAV and EHDV based on VP7 showed more clearly a geographical distribution. The VP7 has been identified as a protein that binds to vector cells (54, 61). The results of the phylogenetic analysis based on VP7 in this experiment, which divided the viruses into three groups (the North American and Australian EHDV-2 and the IBAV groups), might indicate viral adaptability to vector insects.

The nucleotide sequences of segments 6 from the strains of IBAV and CSIRO439 were relatively divergent, as were the sequences of segments 3 and 7. The identities of the deduced amino acid sequences of VP5 among these viruses were more than 98.1% and might bring the

same antigenicity. Although the biological functions of VP5 are not well understood, VP5 was involved in the determination of virus serotype, possibly by imposing conformational constraints on VP2. The higher identities of VP5s from KSB-14/E/97 and the other strains of IBAV and Australian EHDV-2 might give a consistent explanation of the partial cross-reactivity of KSB-14/E/97 to other viruses in the cross-neutralization tests.

Segment 2 displayed remarkable genetic heterogeneity within the EHDV serogroup. The nucleotide sequence identities of segments 2 from KSB-14-E/97 and the existing strains of IBAV were less than 70%, and those of segments 2 from the existing strains of IBAV and the North American EHDV were lower than that. VP2 is the main neutralization antigen of the virus and is the determinant of virus serotype. The low identities of VP2 of KSB-14/E/97 might result in low reactivity in cross-neutralization tests with existing strains of IBAV. The phylogenetic analysis of VP2 and VP5 revealed that KSB-14/E/97 was segregated in a cluster distinct from that of IBAV and Australian EHDV-2. This phylogenetic distinction was remarkable in the tree of VP2. The greatest antigenic and sequence variations are also those exhibited by VP2s of BTV and EHDV (6, 10, 19). It is considered that such low nucleotide identity is the result of reassortment rather than the result of cumulative genetic mutation. The findings of the great variation of VP2, together with the findings of the variations of VP3, VP5, and VP7 of KSB-14/E/97, suggested that this new variant of IBAV was generated by a reassortment event in segment 2 and by mutations, including base substitution, deletion, and insertion, in segments 3, 6, and 7.

In nature, reassortment occurs under the condition that at least two different strains present at the same time, in the same area, and in the same affected host. Although I could not estimate the other parental virus, the new variant of IBAV may have been generated in nature by genetic reassortment between IBAV and another orbivirus.

The results which analyzed RNA segments of four structural proteins indicated that IBAV was clearly segregated from the North American EHDV and showed a slight difference from the

Australian EHDV in nucleotides and amino acid sequences. It may be possible to regard IBAV and Australian EHDV-2 as nearly identical topotype. Little is known about the mechanisms that induce such a variation between IBAV and Australian EHDV. When orbivirus infects host cells, VP2 is responsible for the attachment to mammalian cells, while VP7 mediates the attachment to insect cells (54, 61). Therefore, vertebrate and invertebrate hosts might play important roles in the genetic variations of VP2 and VP7, respectively.

In recent years, epidemics caused by arboviruses have tended to spread over large areas due to the increasing activity of vector insects. Changes in the population of vector insects might be influencing the evolution of the viruses. Further study is needed to investigate the mutation of the virus.

Chapter III

Evidence of an Antigenic Shift among Palyam Serogroup Orbiviruses

SUMMARY

The Japanese isolates of PALV serogroup viruses isolated from 1985 to 2001 were investigated for the genome sequence of segments 2 and 7 and phylogenetically analyzed in comparison with Australian and African isolates of the same serogroup. The nucleotide sequences of segment 7 were highly conserved within Japanese isolates (95.1% to 100%), and between Japanese and Taiwanese isolates (96.0% and 100%), whereas the identities between Japanese and Taiwanese isolates, and Australian and African isolates were fairly conserved (84.2% to 92.0%). Phylogenetic analysis based on segment 7 revealed 3 clusters according to geographical origin. As a result of the nucleotide sequence analysis of segment 2, which encodes a serotype-specific antigen, Japanese isolates were classified into two groups by the genome length and the nucleotide identities. Four of the 9 Japanese isolates were categorized into the same group as prototype strain K-47 of the CHUV, and the remaining isolates were categorized into the same group as the D' Aguilar virus (DAGV) and Nyabira virus (NYAV). Phylogenetic analysis based on segment 2 revealed 2 clusters, the cluster containing CHUV and the cluster containing the DAGV and NYAV. To examine the antigenic relationship among viruses categorized in different clusters, we conducted a cross-neutralization test. KSB-29/E/01 isolated in 2001 in Japan was neutralized by antiserum not only to strain B8112 of DAGV but also to CHUV. These results indicated that genetically and antigenically unique characteristics of KSB-29/E/01 were attributed to genetic reassortment of segments 2 between CHUV and DAGV.

INTRODUCTION

The PALV serogroup virus of genus *Orbivirus* in the family *Reoviridae* is known as an arthropod-borne virus and is distributed in many countries of the African, Australian, and Asian continents (Fig. 1) (29, 30, 58). The D'Aguilar, CSIRO Village, and Marrakai viruses were isolated from *Culicoides* biting midges in Australia in the 1970s (7, 50). NYAV was isolated from an aborted fetus in Zimbabwe in 1973 (53). Although eleven serotypes have been identified in the serogroup by a cross-neutralization test, the pathogenesis of these viruses was not well understood. CHUV was first described as a causative agent of bovine disease characterized by congenital abnormalities with hydranencephaly and cerebellar hypoplasia syndrome in Japan at the outbreak of the disease in 1985-86 (15, 16, 37, 38). The virus was isolated from blood of sentinel cattle and *Culicoides* biting midges at the summer season prior to the disease outbreak and reported as a new serotype distinct from other viruses by a cross-neutralization test (30, 36), but it was reported later that CHUV is cross reactive with Kasba virus, which was isolated in India in 1957 (27). A disease similar to that caused by CHUV occurred sporadically in the southern part of Japan in 1997 and 2001-2002, while the virus was isolated from the blood samples of sentinel cattle and *Culicoides* during the period from 1985 to 2001. The isolates in 2001 seemed to be related to the disease by seroprevalence in sentinel cattle in the epidemic area and the presence of antibodies in the sera of affected calves and their dams. The development of the inactivated CHUV vaccine has made it clear that the characterization of the isolates is essential for the development of preventive measures, including an assessment of vaccine efficacy.

In PALV, VP2 protein encoded by RNA segment 2 is a major neutralizing antigen and has a serotype-specific determinant (23). The VP7 protein encoded by RNA segment 7 is incorporated in the viral inner layer and is a serogroup-specific antigen (62). The sequence analysis of these two segments may reveal the antigenic variation and evolutionary change of the virus.

In this chapter, I examined the Japanese isolates of Palyam serogroup viruses to determine the

genome sequences of segments 2 and 7, and to compare them phylogenetically with Australian and African isolates of the Palyam viruses.

MATERIALS AND METHODS

Viruses and cells. The Palyam serogroup viruses used in this study are shown in Table 3-1. These viruses were propagated in established BHK-21 cells. The BHK-21 cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co.) supplemented with 0.295% tryptose phosphate broth (Difco Laboratories), 0.15% sodium bicarbonate, 2 mM L-glutamine, and 5% calf serum.

RT-PCR of segment 7 and sequencing. Total RNA was extracted from virus culture fluid using High Pure Viral RNA kit (Roche Diagnostics). A portion of segment 7, corresponding to nucleotide positions 258-523 of CHUV segment 7, was amplified via RT-PCR with primers S7-1 (5'-ATCTCAAACCTATAGACCATC-3') and S7-2 (5'-GAACTATTGTTTCCTTGCTGGA-3'). RT-PCR was conducted as described previously (64). PCR products were purified with the QIAquick PCR Purification kit (Qiagen, Valencia, Calif.), and direct sequencing was carried out in both directions with the PCR primers using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and the ABI PRISM 3100 automated sequencer.

Molecular cloning of Segments 2 and sequencing. RT-PCR was performed with the Titan One Tube RT-PCR kit (Roche Diagnostics) containing a proofreading polymerase. The RT-PCR mixture consisted of 0.2 mM deoxynucleoside triphosphate, 5 mM dithiothreitol, 1 × RT-PCR buffer, 1.5 mM MgCl₂, and an enzyme mixture in a total reaction volume of 50 µl. Briefly, the purified viral RNA segments 2 and both strand primers ChuzanS211F (5'-CGCAATGGATGAATTTTCGCTTTGT-3'; 11-35) and ChuzanS2R (5'-GTAAGTGTGTCCCGCAACACG-3'; 3035-3055) for CHUV, and both strand primers PAL2F (5'-GAATTCG

TTAAAATTCCGCAATGGATG-3') and PAL2R (5'-GTAAGTGTGTCCCGCAACACGT AGT-3') for DAGV, were incubated at 94 °C for 5 min and quenched on ice prior to the addition of the reaction mixture. The RT was conducted at 48°C for 60 min. PCR amplification was achieved with initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (95°C, 30 s), annealing (55°C/ 47°C, 30 s, depending on primers), and elongation (68°C, 2 min), with a final extension at 68°C for 7 min. The purified PCR products were ligated into pGEM-T Easy Vector systems (Promega). The recombinant plasmids were transfected into competent *Escherichia coli* JM 109 cells. The positive clones were confirmed by PCR using M13 forward and reverse primers. A plasmid containing the viral genome was obtained with a commercial plasmid extraction kit (Promega) according to the manufacturer's protocol. The 5' and 3' terminal ends of DAGV segments 2 nucleotide sequences were determined using the 5' RACE (Rapid Amplification of cDNA Ends) kit (Invitrogen Corp, Carlsbad, Calif.). Briefly, first-strand cDNA was made using SuperScript II Reverse Transcriptase (Invitrogen) and the primer DAGVSP1 (5'-CGTCATACATTCCCATCTCC-3') for the 5' end and the primer DAGSP1/3Term (5'-AGCTGGATAGGGGCGAATTG-3') for the 3' end, respectively. The reaction was carried out for 60 min at 42°C followed by 15 min at 70°C. Purified cDNA was tailed with dCTP and the terminal deoxynucleotidyltransferase (TdT) and then amplified by the PCR with primers DAGVSP2R (5'-GAATAGGCTTGGGATTATGG-3') and DAGSP2/3TermF (5'-GATGATT CCTATCAAGAGTCC-3'), respectively, and the 5' RACE Abridged Anchor Primer (Invitrogen) under the conditions recommended by the manufacturer. Amplified products were cloned into the pGEM-T Easy Vector. Three individual clones were sequenced in both directions with the Thermo Sequenase Cycle Sequencing kit (U.S. Biochemicals) on a model 4200 automated DNA sequencer (Li-Cor Inc.). Sequences were determined using Universal primers (M13 Forward: 5'-CACGACGTTGTAAAACGAC-3' and M13 Reverse: 5'-GGATAACAATTCACAGG-3') and the segments 2-specific primers as follow: ChuzanS2479F (5'-AGTGGATCCAACGTA

TAGTG-3', 479-498), ChuzanS22560R (5'-TCCGTTAGAAGTGTTTTCTCC-3', 2541-2560), DAGVSP2 (5'-GAATAGGCTTGGGATTATGG-3', 1090-2010), DAGV S23TERM (5'-CATT TGATGAGACAGAGTGG-3', 1686-1705), DAGVS2n3Term (5'-CTTCCTCTACCTTAATTCT G-3', 1738-1757), and DAGVSP2/3Term (5'-GGACTCTTGATAGGAATCATC-3', 2686-2706).

Sequence and phylogenetic analysis. Nucleotide sequences were assembled and analyzed with the GENETYX software (Software Development, Tokyo, Japan). The nucleotide sequences of the ORF region were aligned with the CLUSTAL W program (55). Phylogenetic analyses were carried out by the methods described in Chapter 2.

Sequence data. The sequence data determined in this study were deposited in the DDBJ/EMBL/GenBank database under the accession numbers AB177625 to AB177639, and previously reported nucleotide sequences of CHUV, DAGV, and Australian and Zimbabwean Palyam serogroup virus segments 2 and 7 were retrieved from the database. The GenBank accession numbers are shown in Table 3-1.

Production of bovine antisera. Bovine immune sera against isolate 31 of CHUV and strain B8112 of DAGV were obtained from bovine infected with each virus. Each virus was inoculated intravenously, and sera were collected 4 to 6 weeks after inoculation. Convalescent bovine serum was collected from sentinel cattle naturally infected with isolate KSB-29/E/01.

Cross-neutralization test. Antisera were used to investigate the antigenic relationship among CHUV, DAGV, and the most recent isolate, KSB-29/E/01, by a neutralization test. The neutralization test was performed on 96-well microtiter plates on established HmLu-1 cells. Serially 2-fold diluted serum was mixed with an equal volume of medium containing one hundred 50% tissue culture infectious doses of virus. The mixtures were incubated at 37°C for 60 min, and then HmLu-1 cells suspended in serum-free medium were added to each well. After incubation at 37°C for 7 days in a humidified 5% CO₂ atmosphere, the antibody titer was expressed as a reciprocal of the highest dilution of serum that completely inhibited the cytopathic effect.

RESULTS

Partial sequence and phylogenetic analysis of segment 7. To determine the relationship among the Japanese isolates and other Palyam serogroup viruses, I amplified a portion of segment 7 corresponding to nucleotide position 258-523 by RT-PCR. The PCR product was then sequenced for a portion of 224 bases except for the primer region and compared with the corresponding sequences of Palyam serogroup viruses. Nucleotide identities of segments 7 from 19 isolates or strains ranged from 84.4% to 100%. The identities among the 9 Japanese isolates, K-47, 31, KY-115, ON 91-5, ON-1/E/97, ON-3/E/98, ON-1/E/00, NS-1/P/01, and KSB-29/E/01, ranged from 95.1% to 100%. A high degree of sequence identity, from 96.0% to 100%, was obtained between Japanese isolates and Taiwanese isolates CY-6 and CY-8. The Japanese and Taiwanese isolates showed relatively lower identities (84.2% to 92.0%) to Australian isolates B8112, CSIRO58, CSIRO11, CSIRO82, and DPP66 and to African isolates 792/73, 1726/76, and 1070/78. The deduced amino acid sequences of these 19 isolates showed 100% identity, except for African isolate 1726/76, which showed 98.6% identity to other viruses.

In phylogenetic analysis of the nucleotide sequence of segment 7, these 19 viruses were divided into 3 clusters, which consisted of Japanese and Taiwanese isolates, Australian isolates, and African isolates, respectively (Fig. 3-1).

Sequence and phylogenetic analysis of the full length of segment 2. Because RNA segment 2 encodes the VP2 protein, which is responsible for the serotype-specific antigen, it seems that the full sequence of the genome reflects the antigenic variation of the virus. The cDNA of viral RNA segment 2 was cloned into a plasmid vector and sequenced. The full-length sequence data of isolates were compared with those of Palyam serogroup viruses. The viruses were divided into two groups in lengths coinciding with that of segment 2, that is, with 3055 nucleotides and 3022 nucleotides. Isolates 31, FO88-2, FO90-8, and ON-3/E/98 each have 3055 nucleotides, including an open reading frame of 3006 nucleotides flanked by a 5' noncoding region of 14 bases, a

presumed initiation AUG codon, and a 3' noncoding region of 35 bases. The length of the genome of these viruses was the same as that of prototype strain K-47 of CHUV. Isolates KY-115, ON 91-5, ON-1/E/00, and KSB-29/E/01 each have 3022 nucleotides, including an open reading frame of 2976 nucleotides flanked by the 5' noncoding region of 16 bases and the 3' noncoding region of 30 bases. The same length of genome was cloned from the prototype strain B8112 of DAGV and strain 792/73 of NYAV.

In a comparison of the nucleotide sequence among the Palyam serogroup viruses (Table 3-2), isolates 31, FO88-2, FO90-8, and ON-3/E/98 showed 96.2% to 99.8% identity with each other and 96.4% to 99.9% identity with strain K-47 of CHUV, whereas the identities of these viruses with other Japanese isolates KY-115, ON 91-5, ON-1/E/00, and KSB-29/E/01 ranged from 52.5% to 53.0%. A low percentage of identity was seen in the comparison of these viruses with the prototype strain B8112 of DAGV, 52.4% to 52.7%, and strain 792/73 of NYAV, 52.2% to 52.9%. In contrast, the identities of isolates KY-115, ON 91-5, ON-1/E/00, and KSB-29/E/01 ranged from 95.0% and 99.5% with each other and from 91.9% to 92.9% with the prototype strain B8112 of DAGV and strain 792/73 of NYAV.

The deduced amino acid sequences of VP2 of isolates 31, FO88-2, FO90-8, and ON-3/E/98 showed identities from 97.0% to 99.8% with each other and with K-47 of CHUV, whereas there was less identity with other Japanese isolates and B8112 and 792/73. On the other hand, the deduced amino acid sequences of VP2 of the isolates KY-115, ON 91-5, ON-1/E/00, and KSB-29/E/01 showed high similarity to each other, 94.0% to 98.7%, and with B8112 and 792/73 (Table 3-2).

The phylogenetic analysis of the open reading frame of VP2 revealed that these viruses could be divided into two major clusters (Fig 3-2). The cluster typified by prototype strain K-47 of CHUV contained isolates 31, FO88-2, FO90-8, and ON-3/E/98. The other isolates, KY-115, ON 91-5, ON-1/E/00, and KSB-29/E/01, were included in a cluster together with DAGV strain B8112 and

NYAV strain 792/73, while the cluster was subdivided into 3 minor groups of Japanese isolates, DAGV, and NYAV, respectively.

Cross-neutralization test. To define the antigenic relationship of viruses classified in different clusters by phylogenetic analysis of segment 2, we performed a cross-neutralization test for three viruses, 31, KSB-29/E/01, and B8112, of which isolate 31 was antigenically identical with strain K-47 of CHUV and strain B8112 was the prototype strain of DAGV (Table 3-3). The antiserum to isolate 31 neutralized homologous virus at the titer of 2048; however, the antiserum titers to B8112 and KSB-29/E/01 were lower by 32- to 256-fold. On the contrary, antisera to B8112 and KSB-29/E/01 neutralized homologous viruses and each other at the titer of 512, while the neutralizing titer of these sera to isolate 31 was 16.

DISCUSSION

CHUV was first demonstrated to be the causative agent of bovine disease by Palyam serogroup viruses. Viral infection of a fetus in the uterus results in hydranencephaly and cerebellar hypoplasia syndrome of the newborn calf. The disease was reported in the southern part of Japan as an epidemic in 1985-86 and as a sporadic outbreak in 1997 (15, 16) and 2001-2002. In the most recent outbreak, in 2002, the Palyam serogroup virus isolated from sentinel cattle during the previous year seemed to be related to the outbreak. However, it was suggested that the virus was antigenically different from existing isolates of CHUV, because lower antibody titers to CHUV were detected in affected calves in the later outbreak as compared with those in the previous outbreak. The phylogenetic analysis of genome encoding group-specific and serotype-specific antigens was conducted to define the relationship among the viruses isolated during the period from 1985 to 2001 in Japan and with prototype strains of Palyam serogroup viruses.

The overall identities of nucleotide sequences of segments 7 among Palyam serogroup viruses were higher than 85%, and the deduced amino acid sequence was very similar or identical in all

the viruses examined. The phylogenetic analysis of viral RNA segments 7 revealed that the viruses were divided in different clusters according to the region where the viruses were isolated. The Zimbabwean isolates, 792/73, 1726/76, and 1070/78, which were classified to distinct serotype Nyabira, Gweru, and Marondera, respectively, were categorized in the same cluster. Similarly, the Australian isolates, B8812 of DAGV, CSIRO 11 of CSIRO Village virus, CSIRO 82 of Marrakai virus, and CSIRO 58 of Bunyip Creek virus, were included in the same cluster together with another isolate, DPP 66. All the viruses isolated in Japan were categorized in the same cluster with two Taiwanese isolates. Interestingly, the nucleotides sequence of Japanese isolates taken over 15 years were highly conserved, more than 95%, and the deduced amino acid sequences were identical in all the isolates. These results suggested that the nucleotide sequence of segments 7 of Palyam serogroup viruses were conserved among the viruses in the epidemic area, even if the serotypes were different, and they exhibited the toptotype of viruses. Furthermore, Japanese and Taiwanese isolates of Palyam serogroup viruses seemed to be derived from a common genetic pool.

Segment 2 of viral RNA encodes VP2, which is a serotype-specific antigen (23). Therefore, cDNA of the full genome of segment 2 was cloned and sequenced to investigate its genetic variation among the Japanese isolates and its genetic relationship with other Palyam group viruses. In a comparison of nucleotide identities among the isolates, nine isolates were divided into two distinct groups by the length of genome and by the nucleotide identities. Phylogenetic analysis revealed that the Japanese isolates were divided into two clusters, of which 4 isolates and prototype strain K-47 of CHUV were contained in one cluster and 4 isolates were contained in another cluster with DAGV and NYAV. The identities of deduced amino acid sequences between the two groups of viruses differed by more than 59%, while identity was higher than 94% among the viruses in the same group.

The differences in amino acid sequence between the two groups indicated that the viruses might

exhibit a new antigenic character in a serum neutralization test. The recent isolate KSB-29/E/01 was selected to compare its antigenicity with those of CHUV and DAGV by a cross-neutralization test. The results of the cross-neutralization test demonstrated that KSB-29/E/01 was antigenically close to DAGV. However the virus was neutralized by antiserum to CHUV. This antigenic change of recent isolates of Palyam serogroup viruses might result from a reassortment event in segments 2 between viruses belonging to CHUV and DAGV. Furthermore, the fact that the viruses were isolated in the southern part of Japan indicates that the viruses might invade Japan from the area where both virus groups are epidemic.

In the period from the first isolation of CHUV in 1985 until 2001, a total of 10 Palyam serogroup viruses have been isolated in Japan. During this time, the viruses had not been isolated continuously for two years until 2001 and isolation and seroconversion of Palyam serogroup viruses had not been observed between 1992 and 1996. These viruses are regarded as exotic in Japan. It is possible that several different serotypes of Palyam serogroup viruses have been cocirculated around East Asia and that some of these viruses have repeatedly made incursions into Japan. The coexistence of several viruses in an area might generate reassortant viruses. The KSB-29/E/01, which showed unique antigenicity and was considered to be reassortant, might have emerged under such conditions. Although segment 2 of KSB-29/E/01 had high identity with DAGV and less identity with CHUV, a partial cross-neutralization reaction was observed between KSB-29/E/01 and CHUV. Segment 6 encoded VP5 may be involved with the unique antigenicity of KSB-29/E/01, since segment 6 as well as segment 2 is correlated with serotype specificity on orbivirus.

In conclusion, our data indicated that an antigenic shift occurred in Palyam serogroup orbivirus as a result of reassortment of segment 2. Genetic reassortment appears to have been the main cause of genetic and antigenic diversity of Palyam serogroup orbiviruses. The reassortment event may have contributed to the alteration of viral pathogenicity as well as antigenicity. It is important

to understand genetic and antigenic variation of the viruses circulating in Japan and neighboring areas of East Asia and to develop molecular diagnostic tools and a more effective vaccine for the monitoring and prevention of Palyam serogroup orbiviruses infection.

CONCLUSION

To demonstrate alterations in the pathogenicity and antigenicity of orbiviruses at a genetic level, the genome sequences of orbiviruses isolated in Japan were determined and then analyzed genetically and phylogenetically. In addition, the genetic diversity and evolution of orbivirus isolates in Australia, Africa, North America, and East Asia including Japan, were investigated to aid us in more effectively preventing orbivirus infection in cattle.

One hundred and fourteen field isolates of IBAV were isolated from blood samples of cattle, fetuses, and *Culicoides* biting midges during an outbreak of Ibaraki disease in 1997. Seroconversion was not observed in Japan since last outbreak in 1987. In chapter 1, in order to identify virus isolate, genetic characterization was carried out by RT-PCR-RFLP analysis and antigenic relationship was confirmed by cross-neutralization test. The viruses that induced reproductive problems as well as typical Ibaraki disease among cattle were genetically identical each other and were antigenically closely related but to distinct from previous isolates of IBAV and EHDV-2. The virus was considered to be a putative agent of this outbreak. The results suggest that the virus isolated in 1997 was a variant of IBAV and might be exotic.

The molecular biology of IBAV is poorly understood. Especially, little genetic information was available. In chapter 2, for characterizing genetic diversities of IBAV, the isolate of IBAV was sequenced, and the evolution and phylogenetic relationship of viruses in the EHDV serogroup was investigated. The nucleotide sequences of the isolate's viral RNA segments 2, 3, 6, and 7 were determined and then compared against those of the existing strains of IBAV and EHDV-2. The nucleotide sequences of segments 3 and 7 were conserved, particularly well within the EHDV serogroup among the strains of IBAV and Australian EHDV-2 (less than 8% variability on both segments). Phylogenetic analysis based on the deduced amino acid sequences of segments 3 and 7 revealed that the viruses were differentiated according to their geographical distributions. The results indicated that IBAV and Australian EHDV-2 may share a common genetic pool. The

difference of amino acid sequence may be caused by adaptability to vertebrate and invertebrate hosts. The similarity of the sequence of segment 6 of the isolate to sequences of corresponding segments of the other strains of IBAV and EHDV was found to be approximately 93%. The similarity of segment 2 of the isolate to segments 2 of the other strains of IBAV and EHDV-2 was less than 70%. Phylogenetic analysis based on the deduced amino acid sequences of segments 2 revealed that KSB-14/E/97 was distinguished from clusters of existing strains of IBAV and Australian EHDV-2. These results suggest that the isolates in 1997 were modified by a reassortment of segment 2 and that it exhibits unique genetic and antigenic characteristics. Moreover, pathogenicity in cattle may be altered by reassortment.

In the last chapter, the Japanese isolates of PALV serogroup viruses isolated from 1985 to 2001 were investigated for the genome sequence of segments 2 and 7, and were phylogenetically analyzed in comparison with Australian and African isolates of the same serogroup. The past 16 years by 2001, a total of 10 PALV serogroup viruses were isolated in Japan and seroconversion to PALV serogroup viruses had not been observed between 1992 and 1996. The nucleotide sequences of segment 7 were highly conserved within East Asian isolates (95.1% to 100%), while the identities among East Asian, Australian and African isolates were fairly conserved (84.2% to 92.0%). The deduced amino acid sequences of 19 isolates showed 100% identity, except for African isolate 1726/76, which showed 98.6% identity to other viruses indicated that this region of VP7 were involved in interaction with vector insect. Phylogenetic analysis based on segment 7 revealed 3 clusters according to geographical origin. The result may be reflecting vector competence. As a result of the nucleotide sequence analysis of segment 2, Japanese isolates were classified into two groups by the nucleotide identities. Four of the 9 Japanese isolates were grouped into the same group as prototype strain K-47 of the CHUV, and the remaining isolates were grouped into the same group as the DAGV and NYAV. Phylogenetic analysis based on segment 2 revealed 2 clusters, the cluster containing CHUV and the cluster containing the DAGV

and NYAV. To examine the antigenic relationship among viruses categorized in different clusters, a cross-neutralization test was conducted. KSB-29/E/01 isolated in 2001 in Japan was neutralized by antiserum not only to strain B8112 of DAGV but also to CHUV. These results indicated that genetically and antigenically unique characteristics of KSB-29/E/01 could be attributed to genetic reassortment of segments 2 between CHUV and DAGV.

The present results indicate that orbiviruses isolated in Japan were single topotype and were genetically closely related to other East Asian and Australian isolates. Phylogenetic analyses based on RNA segment 3 or 7 also produced similar results. As a consequence of the reassortment of RNA segment 2, not only minor antigenic change but also alterations in pathogenicity in the cow were observed in IBAV. In addition, an antigenic shift accompanied by serotype conversion occurred in CHUV. It is considered that the reassortment event greatly contributed to such alterations in the viral pathogenicity as well as the antigenicity. When the reassortment event occurred between segments 2, other RNA segments were highly conserved among each of the IBAV and CHUV isolates. These results suggest that orbiviruses circulating in Japan, East Asia and Australia may share a common gene pool and that viruses isolated in geographically different regions evolve independently. Such a circumstance may define the regional distributions of viruses or diseases.

The present study should provide useful information for the improvement of current vaccines as well as the development of molecular diagnostic tools and new preventive measures for related diseases.

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FIGURES and TABLES

TABLE 1-1. Cross-neutralization test among IBAV and EHDV

Virus ^a	Hyperimmune serum against		
	Ibaraki-2	Y87061	KS(H-22)P/97
IBAV Ibaraki-2	128 ^b	64	4
IBAV Y87061	64	128	8
KS(H-22)P/97	8	8	64
EHDV-2 Alberta	8	16	16
EHDV-2 CSIRO439	16	128	8

^a IBAV was isolated from blood of cattle in 1959(Ibaraki-2), 1987(Y87061) and 1997 (KS(H-22)P/97). EHDV-2 Alberta strain was isolated in North America, and EHDV-2 CSIRO439 strain was isolated in Australia.

^b Neutralization antibody titer.

TABLE 2-1. Oligonucleotide primers used for the cDNA amplification and sequencing

Primer	Sequence (5' → 3')	Position	Purpose
L2-1	GTTAAATTGTTCCAGAAATGGAGG	1-24	cDNA amplification
L2-6	GTAAGTTTGTGTTCCAGTAA	2981-3002	cDNA amplification
IBAL2R	GTAAGTTTGTGTTCCAGTAAACC	2978-3002	cDNA amplification
L3F	GTTAAATTCAGAGCGATGGCG	1-23	cDNA amplification
L3R	GTAAGTGTATTTCCAGTCTTTCC	2744-2768	cDNA amplification
IBAM5F	GTTAAAAGGAGGCAC GTTCTTGC	1-25	cDNA amplification
IBAM5R	GTAAGTGAAGGAGTTCGCGTACTC	1617-1641	cDNA amplification
IBAVS7F	GGTAAAATTTGGTGAAAATGG	1-22	cDNA amplification
IBAVS7R	GTAAGTTGAATTTGGGAAAACG	1141-1162	cDNA amplification
M13Forward	CACGACGTTGTAAACGAC		Sequencing
M13Reverse	GGATAACAATTTACACAGG		Sequencing
IBAL2552F	GTTTGCATTTGGCTCAGGAAC	552-571	Sequencing
IBAL22465R	CGCCATCCTGCATGATATTTTC	2227-2246	Sequencing
IBAL21906F	CAAGTGTAACTATCATTACG	1906-1925	Sequencing
IBAL32233R	ACTCGATCGATAGCTGGAGG	2214-2233	Sequencing
IBAL3557F	GGTGCAGCATATATTAGACGG	557-576	Sequencing
IBAL31165F	CCAAAATATGGACGACGACAGC	1165-1184	Sequencing
IBAM5IF	GTCTTATGGCAAAAGTATTGG	495-515	Sequencing
IBAM5IR	GCCAGCAGCCACAAATTCAT	829-848	Sequencing

TABLE 2-2. Nucleotide and deduced amino acid sequence identities of segments 2, 6, 3, and 7 among IBAV and EHDV strains Segment and virus

	% Identity ^a to indicated strain of:					
	IBAV				EHDV-2	
	No.2	Ibaraki-2	Y87061	KSB-14/E/97	CSIRO439	SV 124
<i>Segment 2</i>						
No.2		98.5	96.5	69.4	94.5	70.9
Ibaraki-2	97.8		97.4	69.7	95.3	71.2
Y87061	97.4	98.5		69.9	94.4	71.4
KSB-14/E/97	67.5	67.9	68.2		69.8	68.7
CSIRO439	95.2	96.2	96.4	68.4		71.7
SV 124	72.4	73.3	73.8	67.4	73.8	
<i>Segment 6</i>						
No.2		99.4	97.1	92.6	93.5	
Ibaraki-2	98.3		97.5	92.9	93.9	
Y87061	98.3	99.2		92.2	93.4	
KSB-14/E/97	97.3	98.3	98.7		93.1	
CSIRO439	97.7	98.7	98.7	98.1		
<i>Segment 3</i>						
No.2		97.8	97.7	93.6	92.8	80.5
Ibaraki-2	99.8		99.9	94.9	93.7	80.9
Y87061	99.7	99.9		94.8	93.6	80.8
KSB-14/E/97	99.0	99.2	99.3		92.8	79.8
CSIRO439	98.4	98.7	98.8	98.3		79.8
SV 124	95.6	95.8	95.9	95.3	94.9	
<i>Segment 7</i>						
No.2		98.2	96.2	95.7	92.6	79.9
Ibaraki-2	97.1		97.8	97.3	94.1	81.0
Y87061	97.1	99.4		98.4	93.5	81.0
KSB-14/E/97	97.1	99.4	100		92.8	81.5
CSIRO439	96.0	98.3	98.9	98.9		80.7
SV 124	94.6	96.6	97.1	97.1	96.3	

^a Percentages of nucleotide identity are in lightface; percentages of deduced amino acid identity are in bold face.

TABLE 3-1. Details of Palyam serogroup viruses used in this study

Strain	Serotype	Source	Geographic al origin	Year of isolation	Accession number; Reference	
					Segment 2	Segment 7
K-47	Chuzan	Bovine plasma	Japan	1985	AB014725; (59)	AB014727; (59)
31		Bovine erythrocyte	Japan	1985	AB177637; This study	AB177628; This study
KY-115		Bovine erythrocyte	Japan	1987	AB177636; This study	AB034666; (60)
FO 88-2		<i>Culex</i> spp.	Japan	1988	AB177635; This study	(60)
FO 90-8		<i>Culex</i> spp.	Japan	1990	AB177634; This study	(60)
ON 91-5		Bovine erythrocyte	Japan	1991	AB177633; This study	AB034664; (60)
ON-1/E/97		Bovine erythrocyte	Japan	1997		AB034665; (60)
ON-3/E/98		Bovine erythrocyte	Japan	1998	AB177632; This study	AB177629; This study
ON-1/E/00		Bovine erythrocyte	Japan	2000	AB177631; This study	AB177627; This study
KSB-29/E/01		Bovine erythrocyte	Japan	2001	AB177630; This study	AB177625; This study
NS-1/P/01		Bovine plasma	Japan	2001		AB177626; This study
B8112	D'Aguliar	<i>Culicoides brevitarsis</i>	Australia	1972	AB177639; This study	AB034669; (60)
CSIRO 11	CSIRO Village	<i>Culicoides</i> spp.	Australia	1974		AB034670; (60)
CSIRO 82	Marrakai	<i>C. schultzei</i> & <i>C. peregrinus</i>	Australia	1975	AB034668; (60)	AB034671; (60)
CSIRO 58	Bunyip Creek	Bovine blood	Australia	1976		AB034667; (60)
DPP66		Bovine blood	Australia	1981		AB034672; (60)
792/73	Nyabira	Aborted bovine fetus	Zimbabwe	1973	AB177638 ; This study	AB034674; (60)
1726/76	Gweru	Aborted bovine fetus	Zimbabwe	1976		AB034673; (60)
1070/78	Marondera	Bovine viscera	Zimbabwe	1978		AY078469
CY-6			Taiwan			AY078470
CY-8			Taiwan			

TABLE 3-2. Percentage identities of segments 2 of nucleotide and deduced amino acid sequences among Palyam serogroup viruses

Virus	% Identity ^a to indicated strain of:										
	K-47	31	FO 88-2	FO 90-8	ON-3/E/98	KY-115	ON 91-5	ON-1/E/00	KSB-29/E/01	B8112	792/73
K-47		99.7	99.8	99.9	96.4	52.7	52.7	52.6	52.6	52.7	52.4
31	99.5		99.5	99.6	96.2	52.7	52.9	52.8	52.8	52.6	52.4
FO 88-2	99.6	99.1		99.8	96.3	52.9	52.7	52.5	52.6	52.7	52.2
FO 90-8	99.8	99.3	99.4		96.3	52.7	52.7	52.5	52.6	52.6	52.4
ON-3/E/98	97.4	97.1	97.0	97.2		53.0	52.8	52.6	52.8	52.4	52.9
KY-115	40.3	40.1	40.3	40.3	40.1		95.4	95.0	95.0	92.9	92.5
ON 91-5	40.1	39.9	40.1	40.1	40.0	98.0		98.8	98.7	92.5	92.5
ON-1/E/00	39.7	39.5	39.7	39.7	39.6	97.4	98.6		99.5	91.9	92.0
KSB-29/E/01	39.9	39.7	39.9	39.9	39.8	97.4	98.6	98.7		92.0	92.0
B8112	40.4	40.2	40.4	40.4	39.7	94.9	94.6	94.0	94.0		92.5
792/73	40.2	40.0	40.2	40.2	39.6	96.6	96.3	95.7	95.7	95.9	

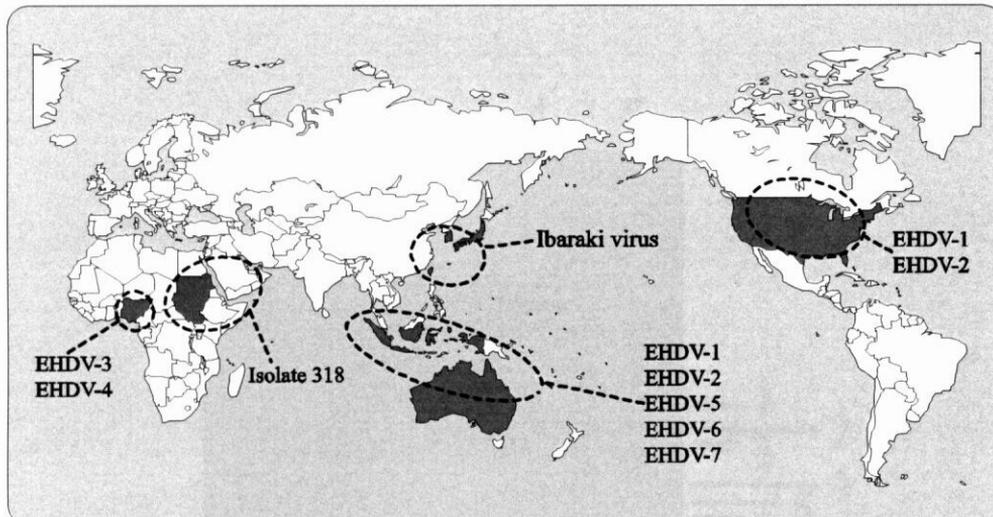
^a, Percentages of nucleotide identity are in lightface; percentages of deduced amino acid identity are in bold face.

TABLE 3-3. Antigenic comparison among CHUV and DAGV strain by cross-neutralization test

Virus ^a	Bovine antiserum to		
	CHUV 31	DAGV B8112	KSB-29/E/01
CHUV 31	2048	16	16
DAGV B8112	8	512	512
KSB-29/E/01	64	512	256

^a, Isolate 31 of CHUV was isolated from the blood of cattle in 1985 in Japan. DAGV was isolated from *Culicoides brevitarsis* in 1972 in Australia. KSB-29/E/01 was isolated from the blood of cattle in 2001 in Japan.

(A) EHDV serogroup



(B) PALV serogroup

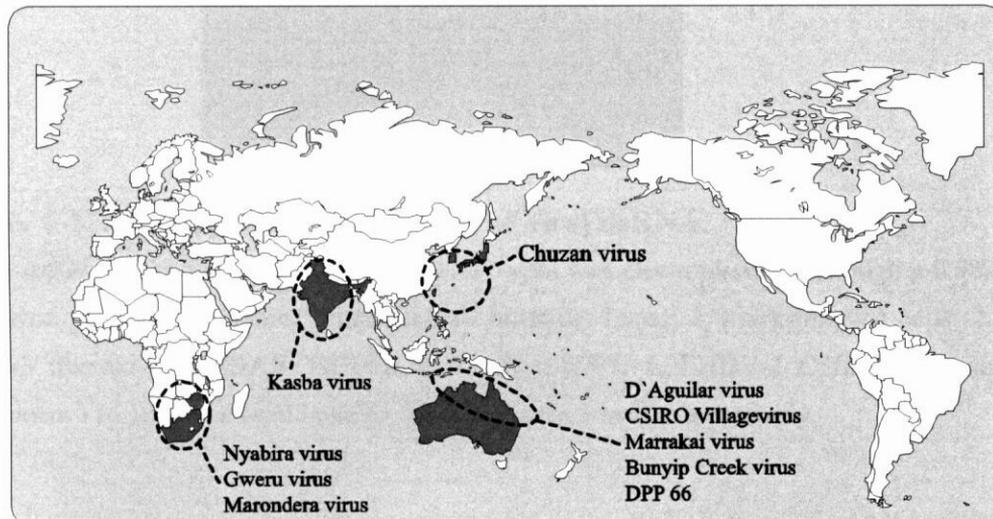


FIG. Global Distribution of (A) EHDV serogroup and (B) PALV serogroup virus.

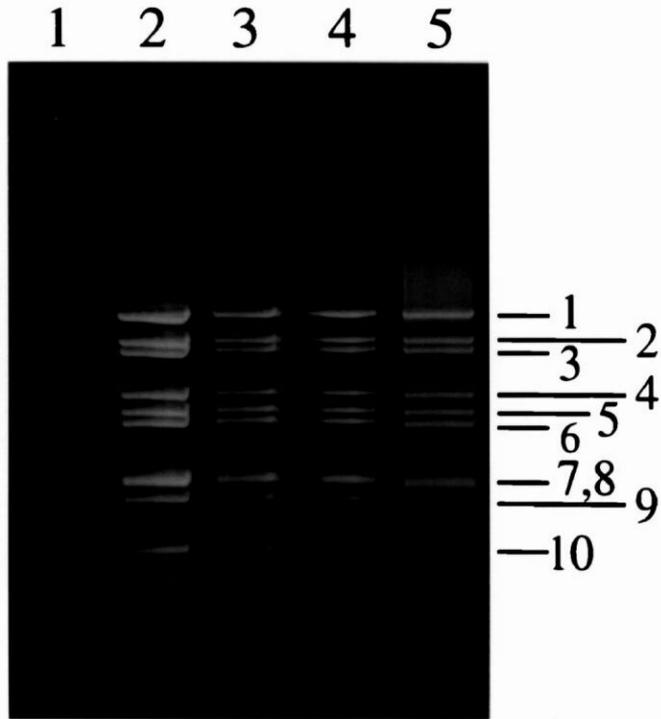


FIG. 1-1. Agarose gel electrophoresis of viral dsRNA.

The dsRNA extracted from infected BHK-21 cells was electrophoresed through a 0.9% agarose gel and was stained with ethidium bromide. Lanes: 1, mock-infected cells; 2, IBAV Ibaraki-2; 3, IBAV Y87061; 4, KS(H-22)P/97; 5, EHDV-2 CSIRO439. The numbers 1 to 10 on the right indicate RNA segments 1 to 10, respectively.

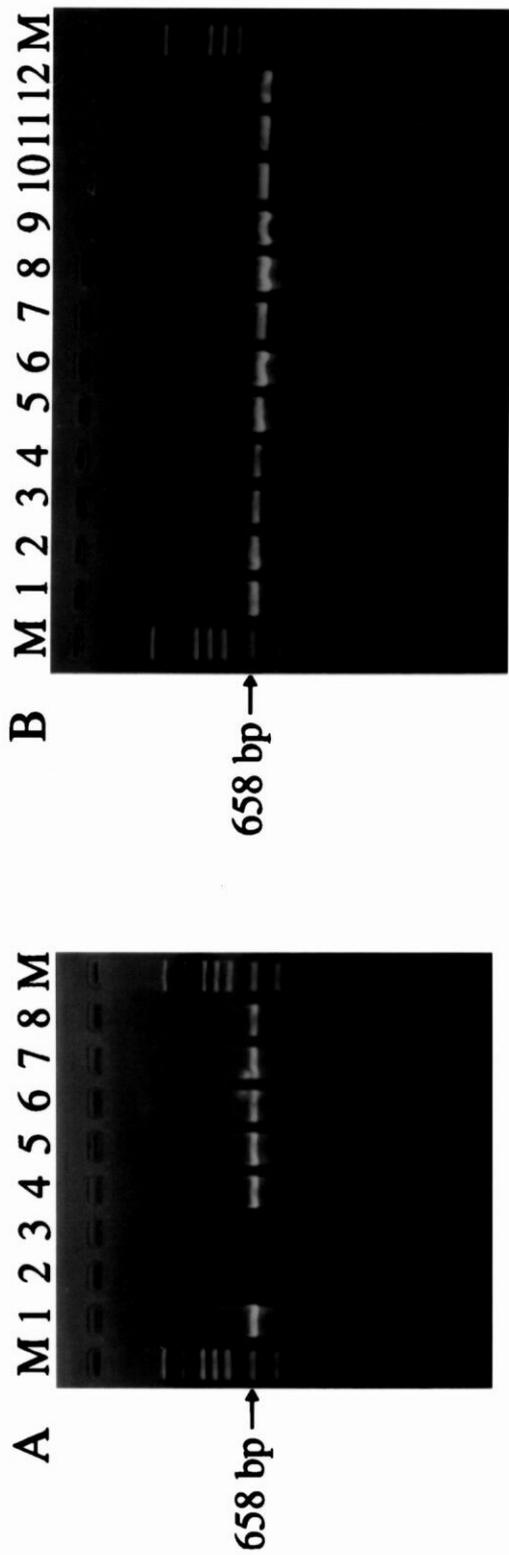


FIG. 1-2. PCR amplification of IBAV and EHDV.

(A) Results of PCR amplification of IBAV strains, North American EHDVs, and Australian EHDVs. Lanes: 1, IBAV Ibaraki-2; 2, EHDV-1 New Jersey; 3, EHDV-2 Alberta; 4, EHDV-2 CSIRO439; 5, EHDV-5 CSIRO157; 6, EHDV-6 CSIRO753; 7, EHDV-7 CSIRO775; 8, EHDV-8 DPP 59; M, pHY marker. (B) Results of PCR amplification of IBAV isolates. Lanes: 1, IBAV Ibaraki-2; 2, IBAV Y87061; 3, isolate from HG; 4, isolate from OY; 5, isolate from FO; 6, isolate from SG; 7, isolate from NS; 8, isolate from KM; 9, isolate from OI; 10, isolate from MZ; 11, isolate from KS; 12, isolate

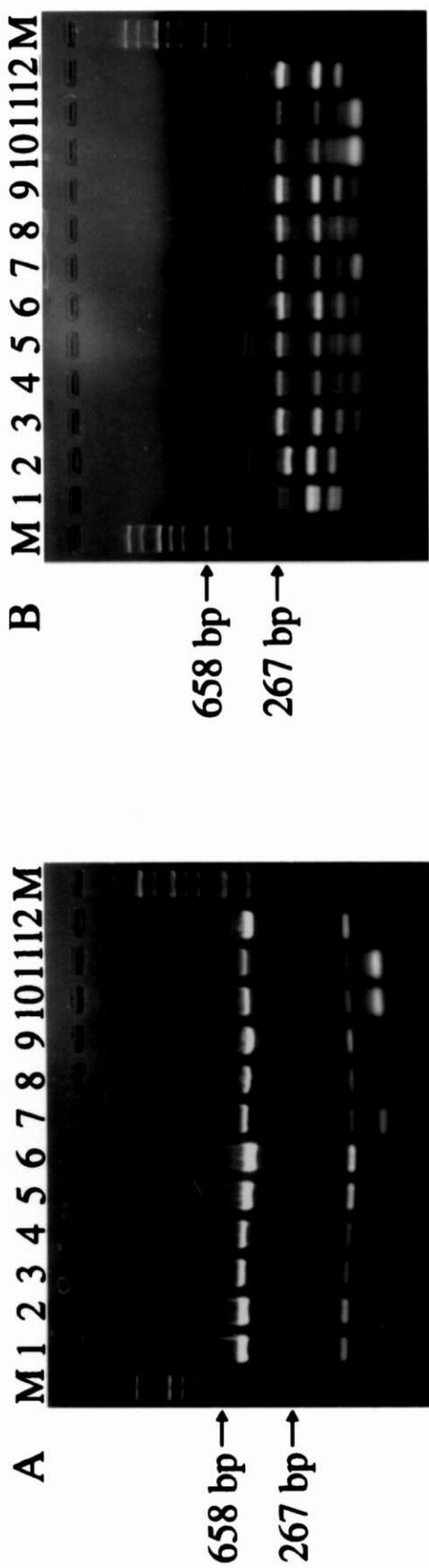


FIG. 1-3. RFLP patterns of PCR products amplified from field isolates in 1997.

PCR products were digested with *Hae*III (A) and *Sau*3AI (B). Lanes: 1, IBAV Ibaraki-2; 2, IBAV Y87061; 3, isolate from HG; 4, isolate from OY; 5, isolate from FO; 6, isolate from SG; 7, isolate from NS; 8, isolate from KM; 9, isolate from OI; 10, isolate from MZ; 11, isolate from KS; 12, isolate from ON; M, pHY marker.

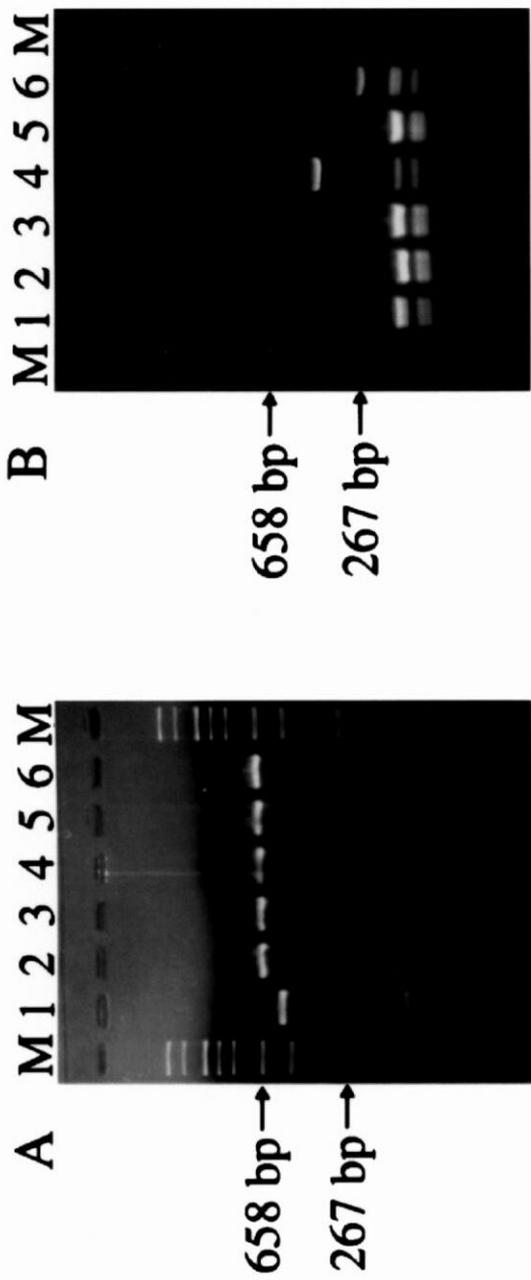


FIG. 1-4. RFLP patterns of PCR products amplified from IBAV and EHDV.

PCR products were digested with *Hae*III (A) and *Sau*3AI (B). Lanes: 1, IBAV Ibaraki-2; 2, EHDV-2 CSIRO439; 3, EHDV-5 CSIRO157; 4, EHDV-6 CSIRO753; 5, EHDV-7 CSIRO775; 6, EHDV-8 DPP 59; M, pHY marker.

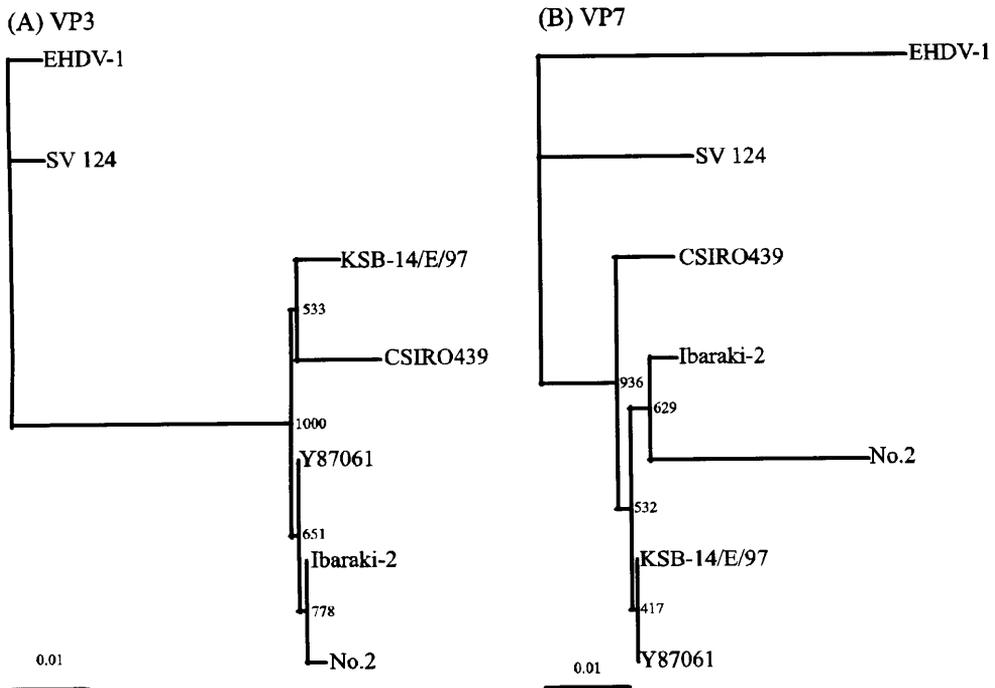


FIG. 2-1. Phylogenetic tree based on VP3 (A) and VP7 (B) of IBAV and EHDV.

Trees were constructed by the neighbor-joining method and bootstrap test ($n = 1,000$). EHDV-1 was used as an outgroup to root the tree. Accession numbers are as follows: EHDV-1 segment 3, X61589; EHDV-1 segment7, D10766; SV 124 (EHDV-2) segment 3, L33819; SV 124 segment 7, AF188643; CSIRO439 (EHDV-2) segment 3, S68010;

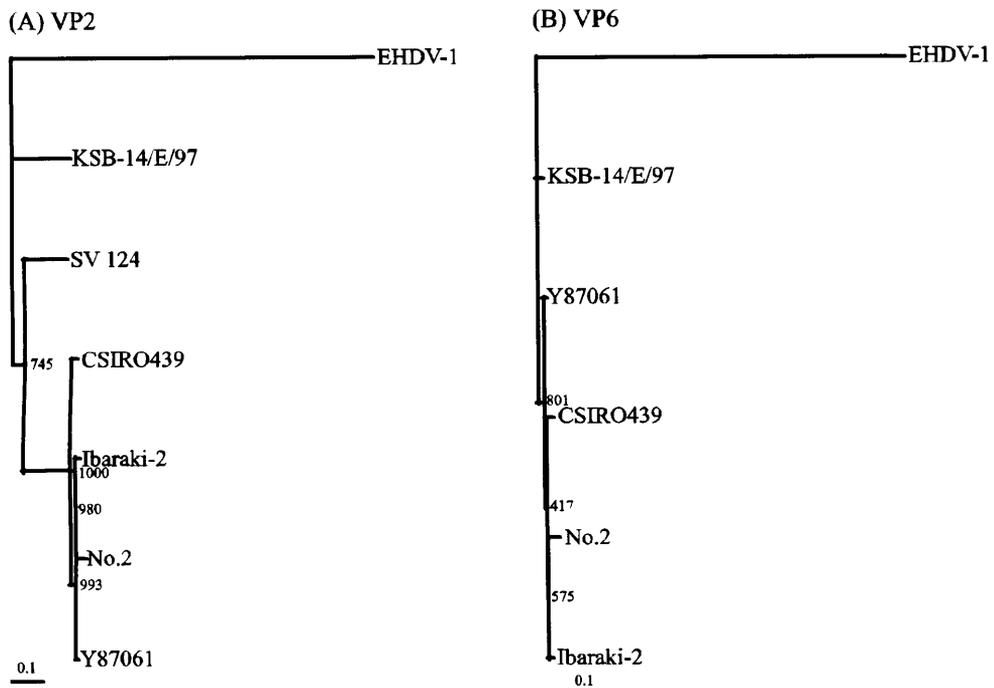


FIG. 2-2. Phylogenetic tree based on VP2 (A) and VP5 (B) of IBAV and EHDV.

Trees were constructed by the neighbor-joining method and bootstrap test ($n = 1,000$). EHDV-1 was used as an outgroup to root the tree. Accession numbers are as follows: EHDV-1 segment 2, D10767; EHDV-1 segment 6, X55782; SV 124 (EHDV-2) segment

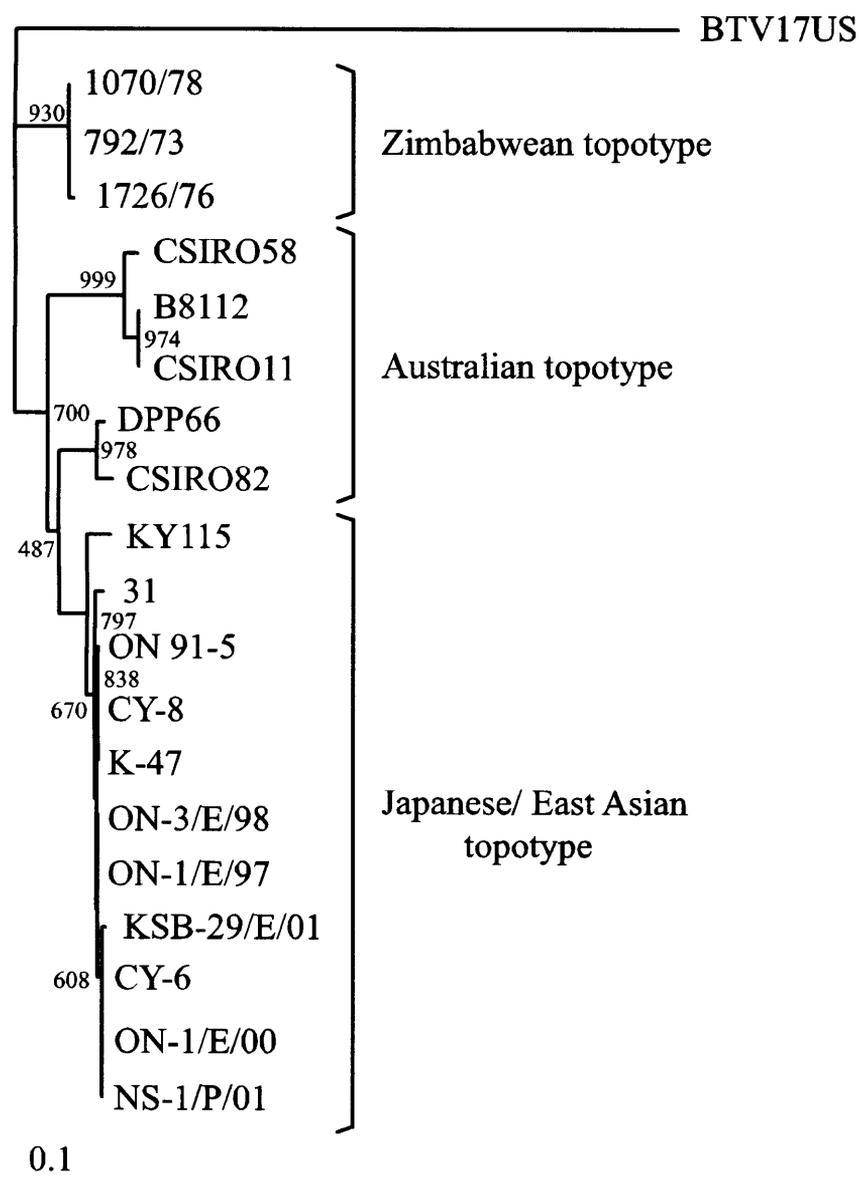


FIG. 3-1. Phylogenetic tree based on the nucleotide sequence of the 224 bp of the segments 7 of the Palyam serogroup viruses from Japan, Taiwan, Australia, and Zimbabwe.

The segment 7 of North American Bluetongue virus serotype 17 was used as the outgroup to root the tree. Numbers above the internal nodes indicate the bootstrap values obtained with 1000 replications.

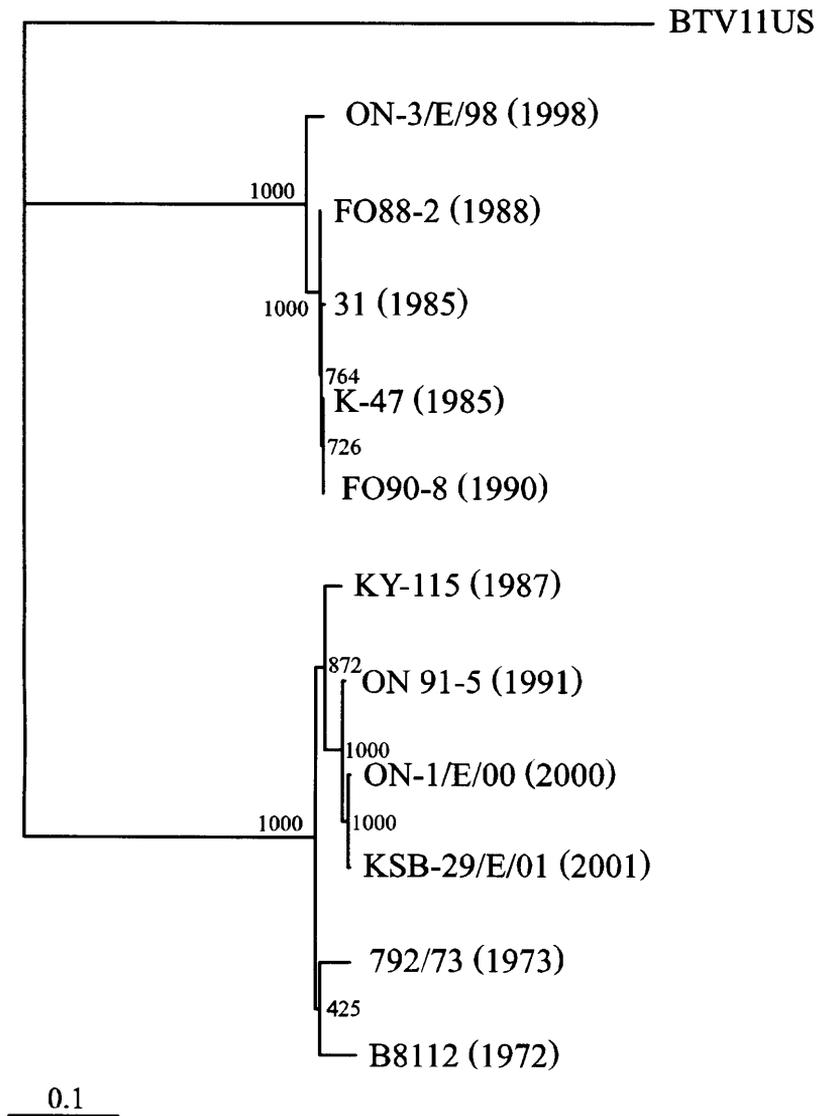


FIG. 3-2. Phylogenetic tree based on the nucleotide sequence of the segments 2 of the Palyam serogroup viruses from Japan, Australia, and Zimbabwe.

The segment 2 of North American Bluetongue virus serotype II was used as the outgroup to root the tree. Numbers above the internal nodes indicate the bootstrap values obtained with 1000 replications.