Characterization of Japanese Isolates of Aujeszky's Disease Virus by Restriction Endonuclease Cleavage Patterns, Virulence in Mice and Thymidine Kinase Activity

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ABSTRACT. Twenty four cloned isolates of Aujeszky's disease virus collected from outbreaks of Aujeszky's disease from 1981 through 1989 in Japan were characterized by their restriction endonuclease (RE) cleavage patterns, virulence for mice and thymidine kinase (TK) activity. All of the isolates belonged to Type II of the four types classified by Herrmann *et al.* (1984). Based on the number and migration rate of the restriction fragments, the isolates were divided into 7 groups with *Bam* HI, 9 groups with *Kpn* I, 3 groups with *Bst*E II and 2 groups with *Sal* I. The results indicate that the RE analysis, especially with *Bam* HI and *Kpn* I, provides useful epidemiological information about field isolates of Aujeszky's disease virus. All of the isolates showed virulence for mice ranging from 6.9 to 63.0 (PFU/LD₅₀). It was interesting that the Nagano S87 strain, which had the highest virulence for the mouse, showed unique RE cleavage patterns with four enzymes. On the other hand, ara-T-resistant, TK-negative strain, was avirulent for mice (>10^{6.4} PFU/LD₅₀). All of the isolates investigated in this study showed TK activity by the thymidine plaque autoradiography.—**KEY WORDS:** Aujeszky's disease virus, cleavage pattern, restriction endonuclease, virulence, thymidine kinase.

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Aujeszky's disease virus (ADV), an α -herpesvirus, causes respiratory distress in fattening pigs, neuropathy and a high mortality in young piglets and abortion in pregnant sows. The first outbreak of Aujeszky's disease (AD) in Japan was recognized in 1981 [11, 18] and the disease spread to Tohoku, Kanto and Kyushu areas.

Several methods have been described for differentiation of ADV isolates based on biological properties such as trypsin- and heat-resistance, ability to form syncytium and virulence for laboratory animals [1, 8, 10, 13, 21, 22]. Restriction endonuclease (RE) analysis of ADV DNA has been used to differentiate ADV isolates which could not be differentiated by other techniques [5, 6, 9, 19, 20]. Nishimori *et al.* [17] reported the easy RE analysis technique using total DNA extract from the infected cells. It was shown that virulence of ADV for pig was correlated to that for mice [12]. In the present study, 24 cloned ADV strains which were isolated from 1981 through 1989 in Japan were analyzed for their RE cleavage patterns and virulence for mice. Furthermore, thymidine kinase (TK) activities of these ADV strains were examined by the thymidine plaque autoradiography method since TK is an enzyme encoded by the gene of herpes viruses including ADV [7] and was indicated as a major determinant of neurovirulence for ADV [25].

MATERIALS AND METHODS

Cells: The following continuous cell lines were employed for virus titration, preparation of viral DNA and TK plaque autoradiography; HmLu-1 cells from baby hamster lung, CPK and PK-15 cells from pig kidney, and Vero cells from African green monkey kidney. The cells were grown in Eagle's minimal essential medium (MEM) containing 5% calf serum and antibiotics.

Viruses and virus isolation: A total of 24 ADV isolates were used in this study and their histories are listed in Table 1. These strains were isolated from 23 pigs or a dog showing clinical signs of AD. The ZT 89–1 and ZT 89–2 strains were isolated from different individuals in the same farm. The other strains were isolated from unrelated farms. The ZKTK[–] strain, which was established from the virulent ZK strain by ara-T treatment [15], was used as the control of TK-negative ADV for TK autoradiography. All viruses were purified three times by the plaque picking method with HmLu-1 cells.

Virus titration: CPK cell monolayers grown in 6-well tissue culture plates (Corning, U.S.A.) at 37°C were inoculated with 0.2 ml volumes of virus suspension, incubated at 37°C for 1 hr and then overlaid with 3 ml of MEM containing 2% calf serum and 1% Bacto agar (Difco). After incubation

Fig. 2. Restriction cleavage patterns with *Kpn* I of the DNA of ADV isolates.

Fig. 3. Restriction cleavage patterns with *BstE* II of the DNA of ADV isolates.

Fig. 4. Restriction cleavage patterns with *Sal* I of the DNA of ADV isolates.

Chiba 61-2 and Chiba 61-796 were similar to lane 3, 4 and 7, respectively.

The RE cleavage patterns of the isolates with *Kpn* I are shown in Fig. 2. The isolates could be divided into 9 groups as shown in Fig. 5. Seven isolates had cleavage patterns similar to lane 3 of Fig. 5, 5 isolates were similar to lane 1, and 4 were similar to lane 7. These 3 major cleavage patterns accounted for 66.6% of the isolates. The remaining isolates showed RE cleavage patterns similar to those of the other 6 lanes. Most of the strains isolated after 1988 showed RE cleavage patterns similar to lane 3.

The RE cleavage patterns of the isolates with *BstE* II are shown in Fig. 3. The isolates could be divided into 3 groups as shown in Fig. 5. Twenty-one isolates had a cleavage pattern similar to lane 1 of Fig. 5 and the isolates showing this cleavage pattern accounted for 87.5% of the isolates. Chiba 62-87

Fig. 5. Schema of the grouping of ADV isolates based on restriction cleavage patterns with *Bam* HI, *Kpn* I, *BstE* II and *Sal* I.

Table 2. Summary of grouping of restriction cleavage patterns with *Bam* HI, *Kpn* I, *Bst*E II and *Sal* I of ADV isolates and virulence for mice

Strain	Endonuclease cleavage pattern ^{a)}				Virulence for mice (PFU/LD ₅₀)
	<i>Bam</i> HI	<i>Kpn</i> I	<i>Bst</i> E II	<i>Sal</i> I	
Yamagata-S81	1	6	1	1	36.3
Hirota	1	1	1	1	46.3
Chiba 58-21	1	6	1	1	20.3
Chiba 58-91	1	1	1	1	12.8
Chiba 59-1	6	3	1	1	50.0
Chiba 59-5	5	7	1	1	33.8
Kanagawa S84	2	1	1	2	20.3
ZK	5	1	1	1	33.2
ZH	5	7	1	1	46.1
Saitama S85	6	1	1	1	28.5
Chiba 61-2	4	7	1	1	45.8
Saitama C86	6	2	1	1	20.7
ZU 86	1	8	1	1	38.1
Chiba 61-796	7	7	1	1	49.5
Nagano S87	3	9	3	2	6.9
Chiba 62-87	1	5	2	1	63.0
Chiba 62-277	1	5	1	1	26.3
Miho	2	3	1	1	19.2
Minori	1	3	2	1	45.2
Ogawa	2	4	1	1	20.2
Saitama 88	1	3	1	1	43.8
Chiba 63-161	1	3	1	1	18.0
ZT 89-1	1	3	1	1	41.3
ZT 89-5	1	3	1	1	45.0

a) Number indicates group of restriction cleavage pattern with *Bam* HI, *Kpn* I, *Bst*E II and *Sal* I shown in Fig. 5.

Fig. 6. Thymidine plaque autoradiography of ADV isolates. Numbers from 1 to 24 are identical with those shown in Table 1. Number 25, the ZKTK⁻ strain; Number 26, mock infection. A: cell monolayers stained with crystal violet. B: autoradiograms after exposure to cell monolayers incubated with [¹⁴C] thymidine for 6 hr. Plaques of TK⁺ ADV show dark rims indicative [¹⁴C] thymidine uptake. Plaques of the ZKTK⁻ strain show TK⁻ phenotype.

and Minori strains were similar to lane 2 and Nagano S87 strain was similar to lane 3 of Fig. 5.

The RE cleavage patterns with *Sal* I are shown in Fig. 4. The isolates could be divided into 2 groups according to whether they had fragment 11 or not. Twenty-two ADV isolates (91.7%) had cleavage

patterns similar to lane 1 of Fig. 5. Among these, fragments 11 and 12 had some molecular weight variation. Only two isolates, Kanagawa S84 and Nagano S87 strains were similar to lane 2 of Fig. 5.

No cleavage site with *Eco*R I existed in any of the isolates.

The RE cleavage patterns of ZT 89-1 and ZT 89-5 strains isolated simultaneously from different individuals in the same farm, were indistinguishable with the four restriction enzymes.

Virulence of the isolates for mice: All of the 24 isolates showed virulence for the ddY mouse. The PFU/LD₅₀ values ranged from 6.9 (Nagano S87) to 63.0 (Chiba 62-87) as shown in Table 2. Nagano S87 strain was 1.9 to 9.1 fold more virulent for the mouse than the other isolates. The ara-T-resistant, ZKTK⁻ strain was avirulent for 5-week-old mice (>10^{6.4} PFU/LD₅₀).

TK activity of the isolates: The TK activity of the 24 isolates shown to be virulent for mice and ara-T-resistant, ZKTK⁻ strain, were examined for their TK activity by the thymidine plaque autoradiography. Plaques made by all of the isolates were labeled with [¹⁴C] thymidine, which indicated that these viruses had TK⁺ phenotypes, while plaques made by the ZKTK⁻ strain were not (Fig. 6).

DISCUSSION

A number of restriction profiles of the field ADV isolates obtained from different areas in Europe and America have been described [2-4, 9, 12, 19, 20, 26]. In this study, DNA of 24 Japanese field isolates of ADV were analyzed by digestion with endonuclease *Bam* HI, *Kpn* I, *Bst*E II, and *Sal* I. These ADV isolates were characterized on the basis of the number and migration rates of the restriction fragments with each endonuclease enzyme. Results obtained showed that 24 ADV isolates were differentiated into 7 groups with *Bam* HI, 9 groups with *Kpn* I, 3 groups with *Bst*E II and 2 groups with *Sal* I. The results of this study indicated that *Bam* HI and *Kpn* I provided more detailed information on the differences between the isolates than either *Bst*E II or *Sal* I.

Herrmann *et al.* [9] classified world-wide isolates of ADV into four (I, II, III and IV) major genome types and five (I, Iv1, Iv2, II and III) genome types with *Bam* HI and *Bst*E II, respectively. According to their classifications, all of the isolates from 1981 through 1989 in Japan were considered to correspond to Type II of the *Bam* HI pattern and Type Iv1 or Iv2 of the *Bst*E II pattern on the basis of the number and the migration rates of the large fragments, and no other types such as Types I, III and IV of the *Bam* HI pattern and Types II and III of the

*Bst*E II pattern were found in the field isolates. Type II viruses in the *Bam* HI pattern were found in Middle Europe [9]. Nishimori *et al.* [17] classified field isolates belonging to Type II into two groups according to the presence (group 1) or absence (group 2) of fragments 8,8' with *Bam* HI. On the basis of that criterion, lanes 1 to 4 and lanes 5 to 7 in Fig. 5 could be classified into groups 1 and 2, respectively. The ADV strains of group 2 were isolated between 1984 and 1986 and not after 1987.

It has been shown that the fragments which show size variation, for example, *Bam* HI fragments 5, 5'+14' and 10 are located in "variable" regions of the genome such as the junctions between the repeat sequences and the short unique sequences, terminus of the long region and an internal area of the unique long region [27]. Minor variation in "variable" regions can occur during serial passage in pigs [16, 27]. It is therefore speculated that ADV was transmitted from Middle Europe to Japan, and minor differences in the migration of relatively small fragments such as those observed in this study occurred during serial passages in pigs in Japan.

ZT 89-1 and ZT 89-5 strains, that were epizootologically related, showed identical RE cleavage profiles with the four restriction enzymes. This shows that RE analysis is useful for epidemiological study of various field isolates. On the other hand, minor variation in RE cleavage patterns could occur during serial passages in pigs [27]. Thus, in some cases, these changes during passage might make it difficult to analyze detailed epidemiological relationships between field isolates by RE analysis.

Laboratory markers and animal models have practical value in helping to evaluate virulence of new field strains. All of the isolates studied in the present investigation showed pathogenicity for the mouse. The levels of virulence (PFU/LD₅₀) of the isolates studies were distributed from 6.9 to 63.0 in mice. Nagano strain (PFU/LD₅₀=6.9) was apparently more virulent than the other isolates. It was demonstrated that virulence for mouse was correlated with virulence for pig [12]. It is therefore possible that Nagano S87 strain is more virulent than the other isolates for pig. In addition, it is of interest that the RE cleavage pattern of Nagano strain was unique among the viruses tested with the four enzymes. Further investigation should be required to define the genetic bases of virulence of ADV.

The TK phenotype is known to affect the expression of virulence of ADV for mice [25] and pigs [15].

In the present study, all of the ADV collected from clinical outbreaks of AD showed TK activity. The findings that TK-positive field strains are virulent for mice and TK-negative, ZKTK⁻ strain, is avirulent, are in agreement with those from the previous investigations [15, 25]. The data presented here indicate that field virulent ADV have TK-positive phenotype.

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