Characterization of Pseudorabies Viruses Recently Isolated in Japan by Restriction Endonuclease Assay

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ABSTRACT. A total of 148 Japanese isolates of pseudorabies virus (PrV) collected in 1987 to 1990 were examined for the cleavage patterns of their genomes by a restriction endonuclease (RE) assay using *Bam*HI and *Kpn*I. Basically, there was no large difference in the cleavage patterns of viruses recently isolated in Japan. All of them were considered as belonging to *Bam*HI cleavage pattern type II as well as strain Yamagata-S81 that is the first isolate of PrV in Japan, suggesting that no remarkable variations occurred in PrVs spreading in Japan since the first outbreak in 1981. However, considerable variations that are probably due to the gain and/or loss of cleavage sites, and to the addition and/or deletion of nucleotide sequences were detected in the repeat, conjunction and left end regions of genome. Some of those variations were similar to one another among the viruses isolated in the same geographical areas or farms at the same times, and from the epidemiologically related outbreaks, indicating that the RE assay on PrV genome is one of useful tools for the epidemiological studies on Aujeszky's disease.—**KEY WORDS:** Aujeszky's disease, DNA cleavage pattern, pseudorabies virus, restriction endonuclease.

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Pseudorabies virus (PrV) is the causative agent of Aujeszky's disease which causes serious economic losses in pig husbandry. Although pigs are considered as the natural host of PrV, the virus can infect occasionally other domestic and wild animals. The severity of clinical signs and mortality in pigs infected with PrV depend on the age of hosts, and a latent infection is established following recovery [10].

The genome of PrV is a linear and double-stranded DNA with molecular weight of approximately 90 megadaltons [2], and composed of the long unique (U_L) sequence and the short unique (U_S) sequence that is bracketed by the inverted and terminal repeat sequences (IR_S and TR_S , respectively) (Fig. 1).

A restriction endonuclease (RE) assay of viral DNAs is a useful tool for the epidemiological studies of Aujeszky's disease, because it can detect genomic diversity of PrV and allow the further differentiation of virus groups [3–9, 11, 12, 15–17]. It is preferable that PrV genome types are classified based on the variation of the regions considered as being relatively stable in the genome. Herrmann *et al.* [11] have classified PrV genomes into four types based on the *Bam*HI RE patterns, and reported that the genome of strain Yamagata-S81, the first isolate of PrV in Japan, belongs to type II which is prominent in the viruses distributing in Middle Europe. The difference between types I and II is whether there is an

additional *Bam*HI cleavage site that yields fragments 2a and 2b in fragment 2 which is considered as a relatively stable region. This appears to indicate that the classification of PrV genome type proposed by Herrmann *et al.* [11] is reasonable. Subsequently, the *Bam*HI RE patterns of other Japanese isolates obtained in 1981 to 1985 have been shown also to be type II, supporting the assumption that PrV that caused the first outbreak of Aujeszky's disease in Japan was brought with swine from a Middle European country [15].

In this study, we investigated 148 Japanese isolates of PrVs obtained in 1987 to 1990 for DNA cleavage patterns, and found that all of them belonged to *Bam*HI cleavage type II, but some showed remarkable variations in the certain regions of genome.

MATERIALS AND METHODS

Viruses and cell culture: A total of 148 PrVs examined were received from the veterinary officials of Aomori (Am), Fukushima (Fs), Saitama (St), Tokyo (Tk), Kanagawa (Kn), Niigata (Nt), Mie (Me), Kumamoto (Ku), Kagoshima (Ks), Ibaraki (Ib), Gunma (Gm), Chiba (Ch) and Tochigi (Tc) prefectures, and were given strain names as follows: name of prefecture and isolation number / later 2 figures of year of isolation (Fig. 2). They contained 140 viruses isolated from pigs and 8 from other

animals species including 2 dogs (Fs02/90 and Kn02/89), 1 cat (Kn04/89), 1 cow (St01/89), 3 domestic minks (Nt01/89, Nt02/89 and Nt03/89), and 1 wild Japanese raccoon dog (Tc09/89). Additional 4 strains, tsG1 [1] and Indiana S [16] from the United States, NK from Thailand, and Yamagata-S81 from the first outbreak in Japan, were used as controls to compare with the recent isolates.

Seed stocks of each PrV isolate were prepared in PK-15 cell culture, which is an established cell line derived from porcine kidneys, using Eagle's minimum essential medium containing 5% bovine serum.

Preparation of viral DNAs: Cellular DNAs extracted from PrV-infected PK-15 cells were used for RE assay. PK-15 cells were infected at a multiplicity of infection of 0.1 plaque-forming unit per cell with each virus. The infected cells were harvested when the cells showed extensive cytopathic effect, washed three times with phosphate-buffered saline and suspended in TE buffer consisting of 10 mM Tris · HCl (pH 8.0) and 1 mM EDTA (pH 8.0). Then sodium dodecyl sulfate and proteinase K were added to cell suspensions to a final concentration of 0.6% and 20 units per ml, respectively, and incubated at 37°C for 3 hr. Cellular DNAs were extracted with a mixture of phenol, chloroform and isoamylalcohol (2:1:0.04) from the infected-cell lysates. The extracts were washed three times with diethyl ether to remove the phenol mixture, added 2 units of RNase A, incubated at 37°C for 1 hr, and then treated again with 10 units per ml of proteinase K at 37°C for 3 hr. DNAs were extracted again with the phenol mixture, washed with diethyl ether, and used for RE assay.

RE assay: The intracellular DNAs prepared from PrV-infected cells were digested overnight with *Bam*HI and *Kpn*I at 37°C. The digested DNAs were subjected to an electrophoresis at 30 volts for 18 hr on a 0.6% agarose gel (195×135×5 mm) containing 1 µg of ethidium bromide per ml in 40 mM Tris-acetate buffer (pH 8.0) supplemented with 2 mM EDTA. After electrophoresis, the gels were observed and photographed under an ultraviolet light illumination.

RESULTS

The *Bam*HI cleavage patterns and physical maps of the DNAs of 4 control PrVs are indicated in Fig. 1. The numbering of each fragment was based on

the report of strain tsG1 [1]. Both strains from the United States, tsG1 (Fig. 1A) and Indiana S (Fig. 1B), were considered as belonging to *Bam*HI cleavage type I, although there was the difference in a cleavage site between fragment 14' and 5'. Strains NK (Fig. 1C) and Yamagata-S81 (Fig. 1D) were classified into type II; they showed the cleavage pattern lacking fragment 2 of type I but appearing two other fragment 2a and 2b that is characteristic of type II.

The cleavage patterns with *Bam*HI of the 148 recent isolates of PrV are shown in Fig. 2. Although there were some variations of the cleavage patterns among the viruses, all isolates showed the similar patterns to one another, and were classified into type II, as strains NK and Yamagata-S81, based on the absence of fragment 2 but the presence of fragments 2a and 2b.

Among the DNA fragments detected in the *Bam*HI RE assay, fragments 3 (16.1 kb), 4 (9.4 kb), 6 (7.5 kb), 7 (6.4 kb), 9 (4.2 kb), 11 (3.2 kb) and 14 (1.4 kb) appeared to be conserved in the all viruses, while the others showed some variations by comparing with the RE pattern of strain Yamagata-S81. The significant variations, that were defined as the large differences of fragment sizes more than 500 bp or changes in the number of bands detected, were observed in fragments 1, 5, 8, 8' and 13', and could be grouped into six types designated as P, A8', A5, D5, D1 and L8 (Fig. 3).

Viruses with the same *Bam*HI cleavage patterns as strain Yamagata-S81 were classified into type P. Type P viruses were most prominent, and 126 isolates (85%) belonged to this type. Viruses classified as types A8' and A5 were possessed of longer fragments 8' and 5 caused by the addition of DNA sequences than type P viruses, respectively. Viruses classified as the type A8' were 7 isolates, Gm13/89, Gm05/90, Ib06/89, Tc13/89, Ch03/89, Ch04/89 and Tk01/89, and type A5 included 4 isolates, Gm27/90, Ib03/88, Ib04/88 and Ib06/88. On the contrary, some isolates showed the deletion of DNA sequences in certain fragments. Eight isolates had shorter fragment 5 resulting from the deletion of DNA sequence than type P viruses, and were classified as type D5. Viruses of the type D5, Tc01/88, Tc08/89, Tc09/89, Tc10/89, Tc11/89, Tc12/89, Tc13/89 and Tc01/90, were found only in Tochigi prefecture. The *Bam*HI fragment 1 of one isolate, Ib14/89, was shorter than that of strain Yamagata-S81, and the virus was classified as type D1. Type L8 viruses showed the

Fig. 1. Physical maps and cleavage patterns of PrV genomes digested with RE *Bam*HI. The top of the figure represents the depiction of PrV genome with map units. PrV genome is organized by unique long (U_L) and short (U_S) regions, and inverted and terminal repeat (IR_S and TR_S) sequences that bracket U_S region. Lines and lanes indicate *Bam*HI RE physical maps and cleavage patterns of the genomes of strains tsG1 (A), Indiana S (B), NK (C) and Yamagata-S81 (D), respectively. Numbering of each DNA fragment was according to the report on strain tsG1 [1].

loss of both *Bam*HI cleavage sites between fragments 8 and 8', and 8 and 13. Two viruses, Ch02/88 and Ch04/88, were included in type L8.

In addition to the significant variations, *Bam*HI fragments 5'+14', 10 and 12 were found to be remarkably variable; the variations of those fragments were detected frequently even in the viruses isolated in the same areas (Fig. 4A). However these variations were expressed as the only small differ-

ences of fragment sizes less than 500 bp, but no changes of the number of bands in the gels.

The *Kpn*I physical map of PrV DNA (Fig. 6) was based on the report of strain tsG1 [1]. The RE patterns with *Kpn*I of PrVs tested were basically similar to one another, although there were conspicuous differences in the length of fragment K among viruses isolated in different areas (Fig. 5). The variation of *Kpn*I fragment K corresponded to that

Fig. 2. *Bam*HI RE patterns of PrVs isolated between 1987 and 1990 in Japan. The names of the individual isolates are indicated on the top of lanes (see text for nomenclature).

Fig. 2. Continued

Fig. 3. Variations observed in the *Bam*HI RE patterns of 148 recent isolates of PrV. P, A8', A5, D5, D1 and L8 show the types of variations, and 126 (85%), 7 (4.7%), 4 (2.7%), 8 (5.4%), 1 (0.7%) and 2 (1.4%) isolates were classified into each type. Dots with numbers marked on the right columns of each lane show conspicuously different bands from those of type P virus.

of *Bam*HI fragment 5 (Figs. 1 and 6). The minor variations were detected in *Kpn*I fragments D, E, F, H, I and J. The variation of fragment D coincided with that of *Bam*HI fragment 5'+14' (Fig. 4 A and B, lanes 1–3, 7 and 8). Other *Kpn*I fragments were found to be conserved in all viruses (data not shown).

DISCUSSION

The diversity of DNA cleavage patterns does not always reflect the antigenic difference of viral components. We have reported previously that there is the antigenic difference in glycoprotein gII of strains Indiana S and Yamagata-S81 [18], but no difference was found in DNA fragment that contains the gene encoding gII of both strains (Fig. 1 and 5, *Bam*HI 1 and *Kpn*I C fragments). On the contrary, the recent isolates of PrV investigated in this report were serologically identical with one another, as determined by reactivity to monoclonal antibodies against glycoproteins gI, gII, and gIII (unpublished data), but the several variations were found in their RE patterns as described above. This indicates the advantage of the RE assay in the classification of PrV, because the assay can allow the further

differentiation of virus groups.

In this study, we tested DNAs of recent isolates of PrVs by the RE assays with *Bam*HI and *Kpn*I, and detected no basic and large differences in their cleavage patterns; all viruses were classified into type II as the viruses isolated before 1985 in Japan. This suggests that no remarkable variation occurred in PrVs spreading in Japan since the first invasion of the virus. Furthermore, the evidence that there were no significant differences among the cleavage patterns of the viruses isolated from pigs and other animals may indicate that the host-dependent mutation of genome type scarcely occurs in PrV. However, some variations were found frequently in both *Bam*HI- and *Kpn*I-digested fragments that involve in the repeat (*IR*_S and *TR*_S), conjunction and left end regions of the genome (Fig. 6), indicating that those regions are relatively variable.

The variations of length of *Bam*HI-digested fragments caused by addition and/or deletion of nucleotide sequences were found in fragments 5, 8', 10 and 12 which related with *Kpn*I fragments K, E, J and I, respectively. These fragments contain the joining sequence of repeat and unique regions. The presence of reiterated nucleotide sequences may account for the variability of joining region. In fact,

Fig. 4. Variations of RE patterns observed in viruses isolated in the same area at the same time, and relationship between those detected in *Bam*HI (A) and *Kpn*I (B) RE assays. Viruses 1 to 3, 4 to 6, and 7 and 8 were isolated in the same areas at the same times, respectively. The relation of variations between *Bam*HI fragment 5' + 14' and *Kpn*I fragment D (lanes 1–3, 7 and 8) was evident, but variations relating to *Bam*HI fragments 10 and 12 were not detected in the *Kpn*I RE patterns (lanes 4–6). Dots with numbers marked on the right columns of lanes indicate DNA fragments that showed variations.

it is known that the end of repeat region which is within *Bam*HI fragment 10 contains two reiterated sequences [19]. *Bam*HI fragment 12 that is part of inverted sequence of fragment 10 also possesses reiterated sequences [19]. Similarly, *Bam*HI fragment 8' includes a part of inverted sequence of fragment 13. It was difficult, however, to detect variation in fragment 13 on the agarose gel. This is probably due to the small size of this fragment.

Although the intensity of bands visualized on the

Fig. 5. Length variation of *Kpn*I K fragment concerned with *Bam*HI 5 fragment. Lane 1; Ib06/88, Lane 2; Ch04/88, Lane 3; Ch06/88, Lane 4; Ch05/88, Lane 5; Tc09/89, Lane 6; NK strain, Lane 7; Yamagata-S81 strain, Lane 8; Indiana S strain, Lane 9; tsG1 strain. Alphabetical figure on the right indicate *Kpn*I-digested fragments concerned physical map shown in Fig. 6.

gel was low, *Bam*HI 5' + 14' and *Kpn*I D fragments that contain the left end sequence of the genome showed remarkable variation; a mobility of those fragments was different even in the viruses isolated in the same area and at the same time (Fig. 4). The low intensity of those bands is probably due to the experimental condition that we used DNA preparations obtained from infected cells in RE assay. It is known that *Bam*HI fragments 5' + 14' and 13, and *Kpn*I fragments D and H disappear when DNA is circularized during its replication. If DNA preparations obtained from mature virions are used for RE assay, more clear bands of those fragments may be detected.

On comparing with strain Yamagata-S81, the variation caused by the loss of *Bam*HI cleavage sites were found only between fragments 8 and 8', and 8 and 13 (type L8). These fragments also contain repeat regions.

It has been reported that variations concerned in the addition and/or deletion of nucleotide sequence occur during experimental passage of PrV in either cell cultures or pigs [13, 14], while there is a no report on the experimental production of variations resulting from the gain and/or loss of RE cleavage

Fig. 6. Location of regions with diversity in the genome of Japanese isolates. The top line represents regions that revealed diversity in *Bam*HI- or *Kpn*I-digested fragments (▨; length variation in *Bam*HI-digested fragments, ▤; length variation in *Kpn*I-digested fragments, ▥; loss of *Bam*HI cleavage site). The middle line indicates *Bam*HI and *Kpn*I RE maps of Yamagata-S81 strain of a Japanese prototype virus. The bottom line represents the genome depicting the U_L and U_S regions as well as the IR_S and TR_S bracketing.

sites by viral passage. It is of great interest that viruses with the later variations, L8 and D1 types, were found in this study. Types L8 and D1 viruses were isolated from pigs in Chiba and Ibaraki prefectures, respectively, of the Kanto district where the outbreaks of Aujeszky's disease are most prominent in Japan. According to Nishimori *et al.*, there were no L8 viruses before 1985, but 5 viruses isolated in the Kanto district in 1985 were all considered as being type L8, although the term of type L8 was not used in their paper [15]. In this study, only 2 (1.5%) of 130 viruses isolated in the Kanto district were type L8. These suggest that type L8 virus appeared in about the end of 1984 and substituted for the prototype virus (type P) in the following year, and thereafter type P virus was again becoming dominant in the Kanto district. However, it was unknown whether those shifts of the RE patterns were due to the invasion of a new virus or to the mutation of the existing viruses. A type D1 virus has been isolated in Aichi prefecture in 1985 [15]. However there was no evidence suggesting the epidemiological relationship between the both outbreaks from which type D1 viruses were isolated in Aichi prefecture in 1985 and in Ibaraki prefecture in 1989. In either case, variation caused by the gain of *Bam*HI cleavage sites seems to be unstable.

Although the variations of the RE patterns were detected only in the repeat, conjunction and left end regions of the genome, the considerable similarity was noted among viruses isolated in the same geographical areas or farms, and from the epidemiologically related outbreaks. For example, the length of *Bam*HI fragments 10 and 12 of the isolates from Niigata (Nt01/89-Nt03/89) and Mie

(Me01/90-Me05/90) prefectures was same in the each virus group (Fig. 2). Furthermore, RE patterns of the viruses isolated from the outbreaks in Mie prefecture were completely consistent with those of Ku01/90 and Ks01/90 that were isolated in Kumamoto and Kagoshima prefectures, respectively. This supports the epidemiological evidence that the affected farms in those prefectures were related to one another by pig trade. In addition, type D5 viruses were isolated only in Tochigi prefecture. These appear to indicate the usefulness of the RE assay for the epidemiological investigations on Aujeszky's disease.

The conclusions obtained in this study are that PrVs spreading in Japan did not show basic changes in the genome type since the first outbreak, but some changes occurred frequently in the certain regions such as repeat, conjunction and left end sequences of the genome.

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