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 BamHI and KpnI. Basically, there was no large difference in the cleavage patterns of viruses recently isolated in Japan. All of them were considered as belonging to BamHI 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.

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 (Uₖ) sequence and the short unique (Uₜ) sequence that is bracketed by the inverted and terminal repeat sequences (IRₜ and TRₜ, 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 BamHI 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 BamHI 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 BamHI 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 BamHI 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 (Mé), 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 (Kk04/89), 1 cow (S01/89), 3
domestic minks (Nt01/89, Nt02/89 and Nt03/89), and 1 wild Japanese raccoon dog (Te09/89). 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 mini-
imum essential medium containing 5% bovine
serum.

**Preparation of viral DNAs:** Cellular DNAs ex-
tracted from PrV-infected PK-15 cells were used for
RE assay. PK-15 cells were infected at a multiplica-
tion 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 protease K were
added to cell suspensions to a final concentration of
0.6% and 20 units per ml, respectively, and incu-
badated 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 protease
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
BamH1 and Kpn1 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 BamH1 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 BamH1
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
the other two fragment 2a and 2b that is characteristic of
type II.

The cleavage patterns with BamHI 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
BamH1 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 all viruses,
while the others showed some variations by compar-
ing 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 BamHI 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 clas-
sified 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, Tb13/89, 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 frag-
ment 5 resulting from the deletion of DNA sequence
than type P viruses, and were classified as type D5.
Viruses of the type D5, Tb01/88, Tb08/89, Tb09/89,
Tc10/89, Tc11/89, Tc12/89, Tc13/89 and Tb01/90,
were found only in Tochigi prefecture. The BamH1
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
loss of both *BamH*I 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, *BamH*I 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 differences of fragment sizes less than 500 bp, but no changes of the number of bands in the gels.

The *KpnI* physical map of PrV DNA (Fig. 6) was based on the report of strain tsG1 [1]. The RE patterns with *KpnI* 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 *KpnI* fragment K corresponded to that
Fig. 2. *BamHI* 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
of BamHI fragment 5 (Figs. 1 and 6). The minor variations were detected in KpnI fragments D, E, F, H, I and J. The variation of fragment D coincided with that of BamHI fragment 5'+14' (Fig. 4 A and B, lanes 1-3, 7 and 8). Other KpnI 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, BamHI I and KpnI 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 BamHI and KpnI, 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 BamHI- and KpnI-digested fragments that involve in the repeat (IRs and TRs), conjunction and left end regions of the genome (Fig. 6), indicating that those regions are relatively variable.

The variations of length of BamHI-digested fragments caused by addition and/or deletion of nucleotide sequences were found in fragments 5, 8', 10 and 12 which related with KpnI 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,
it is known that the end of repeat region which is within *BamHI* fragment 10 contains two reiterated sequences [19]. *BamHI* fragment 12 that is part of inverted sequence of fragment 10 also possesses reiterated sequences [19]. Similarly, *BamHI* 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 gel was low, *BamHI* 5' + 14' and *KpnI* 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 *BamHI* fragments 5'+14' and 13, and *KpnI* 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 *BamHI* 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 no report on the experimental production of variations resulting from the gain and/or loss of RE cleavage
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 BamHI 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 BamHI fragments 10 and 12 of the isolates from Niigata (Nt01/89-Nt03/89) and Mie (Me01/90-Me05/90) prefectures was same in 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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