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MOLECULAR COMPARATIVE STUDY ON DIFFERENT STRAINS OF EQUINE HERPESVIRUS TYPE 1 (EHV-1) BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

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ABSTRACT

RacL11 and Kentucky D are classical virulent strains of equine hepesvirus-1 (EHV-1) which are worldwide used for vaccine manufacture. These two strains are the parent strains of commercial vaccines. The present study were applied in the Laboratory of Veterinary Microbiology, Faculty of Applied Biological Sciences, Gifu University, ,Japan in 2006. We compared the two strains with the reference strain Ab4P using Long and accurate PCR (LA-PCR) Restriction Fragment Length Polymorphism (RFLP) for the EHV-1 whole genomic DNA using eight different types of restriction enzymes (SacI, SspI, NaeI, ApaI, EcoRI, HincII, BglII, and StuI) which digest the whole genome into 600 fragments. Digests were electrophoresed on an agarose gel of the appropriate concentration. Restriction maps of RacL11 and Kentucky D genomic DNA in comparison to Ab4p

revealed no restriction sites differences except in one fragment (Fragment 12) which corresponds to nt 104,320 to 111,287 in the genome sequence of the standard Ab4p strain and includes a part of ORF62, the intergenic region between ORFs 62-63, and a portion of ORF63. and by sequencing we found that the intergenic region between ORFs 62-63 (IGR) revealed that the RacL11 and Kentucky D strains show sequence length difference between all examined strains. The increased length of fragment 12 of RacL11 was found to be due to the presence of 10 copies of 5'-GGCTAGCGCTAACGCTAG-3' while the Kentucky D and Ab4p sequences contained 4 copies and 3 copies, respectively. Using more restriction enzymes for future RFLP comparison is recommended.

Keywords: EHV-1, RFLP, DNA, Genomic differences

INTRODUCTION

EHV-1 causes abortion, respiratory disease, neonatal deaths and neurological disorders in horses ***(Allen and bryans, 1986).*** The EHV-1 genome has been characterized as a linear double-stranded DNA molecule. The complete DNA sequence of a British isolate, Ab4p, has been determined ***(Telford et al., 1992).*** The genome is 150,223 bp containing 80 open reading frames. Since four open reading frames are duplicated, the genome contains at least 76 distinct genes. Researchers classified various field strains of EHV-1 according to restriction site mapping, plaque characteristics in cell cultures and clinical manifestations in experimentally infected hamsters ***(Doll et al., 1954; Hübert et al., 1996; Pagamjav et al., 2005; Studdert et al., 1992).***

There are at least two major groups of EHV-1 based on DNA electropherotype patterns, designated type P and type B ***(Allen et al.,1985; Allen and Bryans, 1986).*** The majority of archival strains from aborted fetuses in Kentucky prior to 1981 were type P. However, by 1983 type B had displaced P as the predominant cause of abortion. In Canada and Australia, type B has also been isolated from aborted fetuses ***(Nagy et al., 1997; Studdert et al., 1992).*** In Japan, although type P accounted for the majority of isolates from aborted fetuses, type B virus was first isolated from an imported pregnant mare in 1994, and type B virus has been isolated occasionally since 1997 and has caused epidemics of abortion in the same district in Japan. EHV-1 P and B genotypes were found to be differentiated by RFLP ***(Matsumura et al., 1992).*** The virulence of the B and P types of EHV-1 Japanese isolates in hamsters were previously tested ***(Pagamjav et al., 2005)*** and it was found that all EHV-1 B strains were non virulent while EHV-1P strains were virulent, mildly virulent or non virulent. The determinants of virulence and pathogenicity of EHV-1 various isolates are still unknown and need to be investigated. ***Allen et al. (1985)*** found that, by restriction endonuclease characterization of the DNA of 317 isolates of equine abortion virus (EHV-1) from 176 epizootically unrelated outbreaks of equine virus abortion occurring over 24 years in Kentucky, an epizootic pattern difference and variation of the virus have emerged. ***Matsumura et al. (1992)*** reported the homogeneity of EHV-1 isolates in Japan, showing all of EHV-1 isolates in Japan examined had the same DNA fingerprints after digestion with *Bam*HI, *Bgl*II, and *Eco*R1 with slight variations in repeat sequences. We attempted to identify DNA genomic differences

between RacL11 and Kentucky D with that of Ab4p by restriction site mapping analysis. RacL11 and Kentucky D, both strains are wild type virulent strains of EHV-1 that were isolated from aborted horse fetuses by newborn hamster inoculation ***(Studdert et al., 1992).*** These two strains are parent strains of commercial vaccines. RacL11 was isolated in Poland and Kentucky D was isolated in USA and both of them are P genotype. Strain RacL11, a highly virulent isolate was obtained from an aborted foal ***(Mayr et al., 1968).*** It was found that the annual incidence of EHV-1 abortion in Kentucky's thoroughbred broodmare population due to Kentucky D strain started in 1957 until the present time, and the incidence was very high from 1957-1981 until wide spread vaccination of the disease began in 1981 ***(Allen and Bryans, 1986; Hübert et al., 1996).*** The purpose of this study is the Investigation of the cause of the higher pathogenicity of RacL11 and Kentucky D as wild type virulent strains of EHV-1 in hamster model. Little information is available about the correlation between genomic structure and expression of specific pathogenicity in various EHV-1 strains. Identification of the DNA genomic differences between wild type virulent strains of EHV-1 RacL11 and Kentucky D (found to be hepatopathogenic in hamster model) with that of Japanese field horse isolates and Ab4p (found to be non hepatopathogenic in hamster model) strains of equine herpes virus type 1 (EHV-1). Identification of gene(s) which may play some roles in inducing hepatopathogenicity in hamsters inoculated by RacL11 and Kentucky D strains of EHV-1 by restriction fragment length polymorphism (RFLP).

MATERIALS AND METHODS

Equine Herpesvirus-1 strains:

EHV-1 strains used in this study were RacL11, Kentucky D and Ab4p. Ab4p was kindly provided by Dr. A. J. Davison, Glasgow University, UK. RacL11 and Kentucky D were kindly provided by Dr. N. Osterrieder, Cornell University, USA. RacL11, Kentucky D, Ab4p were passaged twice in fetal horse kidney (FHK) cells in the Laboratory of Veterinary Microbiology, Faculty of Applied Biological Sciences, Gifu University, ,Japan after arrival and the strains were propagated as described previously ***(Pagamjav et al., 2005)*.**

Virus propagation and DNA extraction:

Rabbit Kidney (RK13) cells were grown in Eagle's Minimum Essential Medium (Eagle's MEM) (Nissui, Tokyo, Japan) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 5% fetal bovine serum (FBS). Viruses were propagated by inoculation in fetal horse kidney cells (FHK) at low multiplicity of infection (moi) 0.01. Total DNA was extracted from infected FHK cells when about 90% of the cells showed the cytopathic effect (CPE) by phenol chloroform method (previously described ***(Desai, et al., 1993)***. DNA concentration and purity was evaluated with Gene Quant II (Amersham Pharmacia, Tokyo, Japan).

PCR Amplification:

The PCR amplification primers were designed (Table 1) and sent to Dragon Genomics (Yokkaichi, Mie, Japan). The PCR reaction mixture and PCR program were standardized to specifically amplify each

genomic fragment without presence of any unspecific PCR product as follows. Each genomic DNA sample was amplified by PCR using 1 µ1 of DNA in 50 µ1 reaction mixture consisting of 13.5 µl distilled water, 8 µ1 dNTP mixture, 25 µ1 2x GC buffer 1, 1 µ1 of each primer(100 pmol), LA-Taq enzyme 0.5 µ1 (TAKARA Bio INC., JAPAN). The amplification program was denaturation at 95 ^{0}C for 1 minute followed by 30 cycles of denaturation at 94 ^{0}C for 20 seconds, annealing at 53 ^{0}C for 30 seconds and extension at 72 ^{0}C for 45 seconds (TAKARA Biomedicals.). Each Amplified product was precipitated by ethanol, resuspended in 30 µ1 of T.E. buffer and stored for at least 24 hours at 4 ^{0}C until use. DNA was quantified by gel electrophoresis using a 100 bp DNA ladder markers (TAKARA Bio INC., JAPAN- code no. 3407A) in 0.9%.agarose gel Tris.

phosphate –EDTA buffer:

Restriction mapping and sequencing. Total DNA was extracted from infected FHK cells by the phenol chloroform method as described previously**.** Long and accurate PCR (LA-PCR) for the EHV-1 genome was performed as described previously ***(Pagamjav et al., 2005).*** The LA-PCR products were digested with eight restriction enzymes (*Sac*I, *Ssp*I, *Nae*I, *Apa*I, *Eco*RI, *Hin*cII, *Bgl*II, and *Stu*I). Digests were electrophoresedon an agarose gel of the appropriate concentration. DNA sequencing was examined by Dragon Genomics Inc.,Mie,Japan. Computer programs,GENETYX-MAC/ATSQ, GENETYX –MAC, phredPhrap and consed were used for DNA sequence assembly and analyses.

Table (1): Primers used for LA-PCR RFLP. Eighteen pairs of primers. Fragments.

Primer	Position	Sequence	Tm ºC	GC%
0-F	831- 858	TGTCCATCGTTCTGAAACACCCATTGCC	64.0	50.0
1-F	2,227-2,306	GCTTGAGGTGGCGGAACTTATGCGGGTGTACTCCT	71.2	57.1
1-R	15,034-15,004	ACGCCCACTCCCACCACTACCTCCTATTCAC	69.4	58.1
2-F	12,042-12,074	TCTGCTCGCGCTCTCCATCTCTCACCACACTCA	70.3	57.6
2-R	24,225-24,193	CAGCCAGATAGAGAGCCCGGTGAAGACTCGTGT	70.3	57.6
3-F	22,072-22,104	CTCGCGGGCATTTTTGTACCACCGCAGCTTGAC	70.3	57.6
3-R	34,265-34,233	AGCGCCCCCGAGGTTAAGATTTCAGCGTCCGTT	70.3	57.6
4-F	30,926-30,961	GGTCCGCTGAATAAAGTCTAGGTGGTCGAGGATGTC	69.9	52.8
4-R	41,177-41,145	CTTTGCGGCTTCCACCACCTCGCGCAGAATATG	70.3	57.6
5-F	41,139-41,169	GCTACCCATATTCTGCGCGAGGTGGTGGAAG	69.4	58.1
5-R	51,877-51,841	TAACCAAGACCGCACGCCCCCAACCTAAACGCAACT	70.8	54.1
6-F	50,572-50,604	CGCCACAACCTCCCGCTCATATCTCTCGAACAC	68.8	51.4
7-F	60,313-60,346	GGCACTATTCGGACGCACCCTAGACCACTTTGAC	70.2	55.9
7-R	74,673-74,641	CTGAAGCGCGGGGGTGTAACTGTGCATGTAAGC	70.3	57.6
8-F	70,217-70,249	GCCGCTTTACCACGCCCTACCTTATCCTACACG	70.3	57.6
8-R	82,519-82,487	GCTTGCGCGAGTGGAACGGTCAGCGGAAATAGT	70.3	57.6
9-F	80,772-80,805	CTGATGGCGGGGTACTTCAAACTCGGGCCAATAG	70.2	55.9
9-R	91,710-91,676	TGGTAGACGCGGTAGCCAATCCACGCCGAAGCAAA	71.2	57.1
10-F	90,893-90,927	TTTCCCACCACCCCCTTGACGACTATCTATCTACGGCAT	70.3	54.3
10-A	98,562-98,524	ACAGATGGCAAAACCCCTCTGATTGACTTCGTGCTCCAC	70.5	51.3
10-B	97,018-97,053	CAGGTTTACGGAGCCGTTGACTTCGACGCACTGTAA	69.9	52.8
10-R	105,616-105,583	TCCCCCTCCTGGTCGGCGTATAAGGGATCTGACT	71.4	58.8
11-F	104,320-104,354	CTACGAGTTTGTGAGACCAGAGACCACGCAGGAGG	71.2	57.1
11-R	111,287-111,251	CCGACCCAGAAGATACGAGCGATGAAACCAGCACAGA	70.8	54.1

Primer	Position	Sequence	Tm ºC	GC%
12-F	108,665-108,696	CAGTTCGAGCCTAGCCCAGCAGCGCATAAACC	70.5	59.4
12-R	113,940-113,902	GGCGTTGTATCTAGCAGCCCACGTTCCTTATTGCTCACA	70.5	51.3
13-F	113,776-113,808	ACCCGCCCATCAACCCGCCCAGTAAACAAAGAC	70.3	57.6
13-R	122,136-122,100	GACTGCGTATCGCTTTCGCTGCTAGACCACT	70.8	54.1
14-F	119,389-119,421	TTTCGCCTCCCCCCCGCTCCAACCAATTAGAAC	70.3	57.6
14-R	130,157-130,125	CGTGGTCGTAGCAGAGGTAGTCGAGGCTGAAAC	70.3	57.6
15-F	125,251-125,283	TGTTGTGCAGGGCTAGGCGGTGGTGGGTGTAAA	70.3	57.6
15-R	139,895-139,861	ATGCTCTTCTGCGCGTACTGGTGCTGCCTGGGACA	72.4	60.1
A-16-F	135,545-135,583	CCTCGGGCACAAAATCATCACTCCTCATCCATCTGTAGC	70.5	51.3
A-16-R	146,028-145,996	CTCTCGAACACCATGCGGACCAGAGCGTTGTAC	70.3	57.6
17-F	144,025-144,059	GCTCTTGGCACTCCTTCTTCGGCTTGCGGAGGTAA	71.2	57.1
17-R	4,344-4,310	CGTTGTGTCGCCTCTGCCGCTTGACCTGACTTGGA	72.4	60.1

RESULTS

Restriction fragment length polymorphism (RFLP):

A comparison of about 600 restriction sites in RacL11, Kentucky D, and Ab4p genome showed no significant differences, although the LA-PCR of fragment 12 was longer in RacL11 than in Kentucky D and Ab4p. The LA-PCR of fragment 12 corresponds to nt 104,320 to 111,287 in the genome sequence of Ab4p and includes a part of ORF62, the intergenic region between ORFs 62-63, and a portion of ORF63. The increased length of fragment 12 of RacL11 was found to be due to the presence of 10 copies of 5'-GGCTAGCGCTAACGCTAG-3' while the Kentucky D and Ab4p sequences contained 4 copies and 3 copies, respectively (Fig. 1,2,3).

Fig. (1): LA-PCR amplification products of each genome of RacL11, Kentucky D and Ab4P by Agar gel electrophoresis.

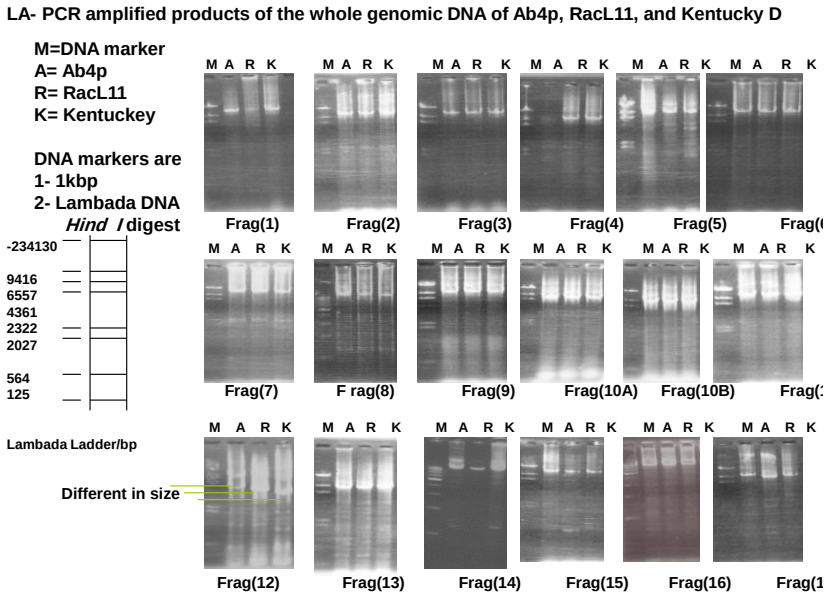


Fig. (2): LA-PCR amplification products of each genome of RacL11, Kentucky D and Ab4P by Agar gel electrophoresis.

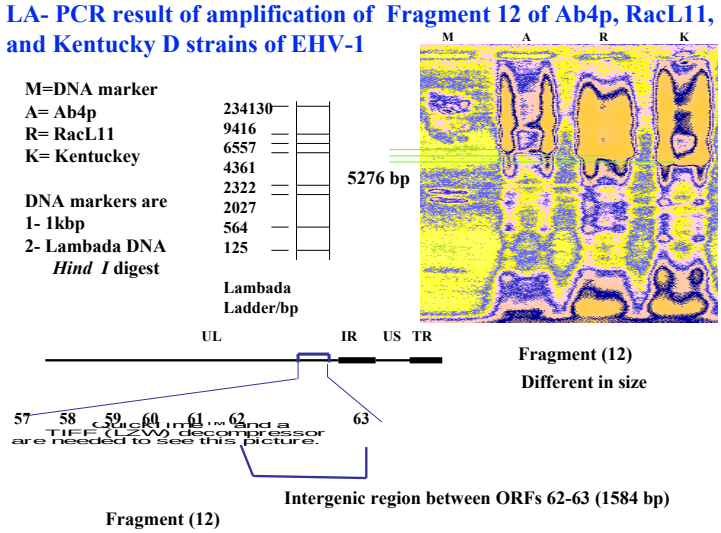
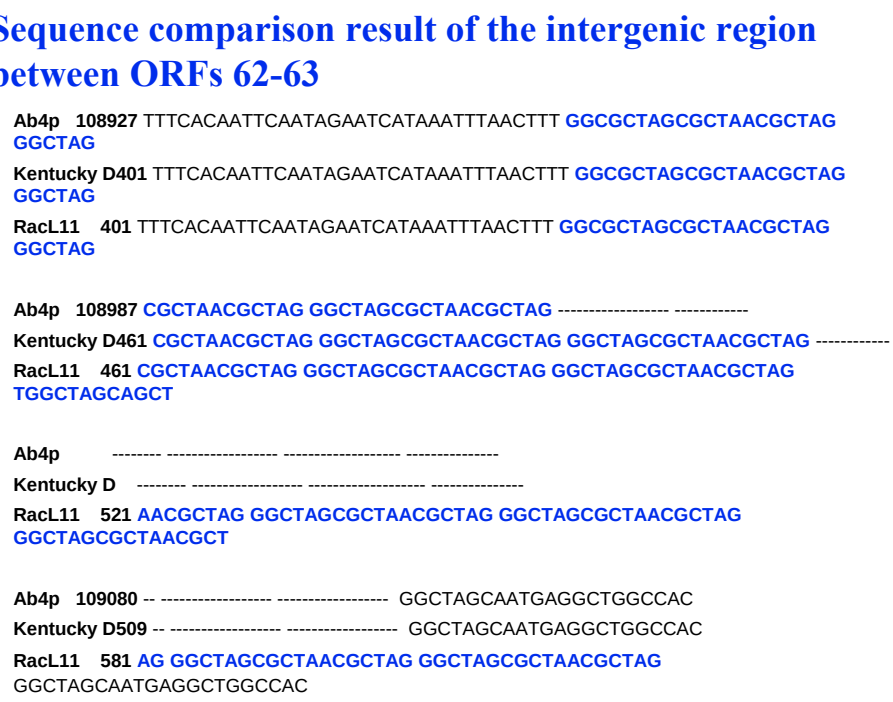


Fig. (3): Sequence comparison showing difference in length of the intergenic area between ORF 62-63.



DISCUSSION

Previous studies have utilized DNA restriction fragment length polymorphism (RFLP) to separate field isolates of EHV-1 into subgroups according to characteristic restriction enzyme site changes and the presence of variable numbers of copies of short sequence repeats. These studies demonstrated a relatively low frequency of genetic polymorphism for EHV-1 and suggested that distinct strains of EHV-1 do exist in the field ***(Allen et al., 1985; Binns et al., 1993; Goodman et al., 2007; Guanggang et al., 2010; Osterrieder N, Davis-Poynter N McCann et al., 1995; Palfi and Christensen, 1995; van Maanen et al., 2001).***

However, the relative lack of variation of EHV-1 sequences between strains has resulted in too few RFLP variants to be identified for detailed epidemiological studies. Furthermore, although such analyses may be used for tracing the genetic relatedness of strains, they allow identification only of those genetic changes resulting in restriction fragment variation, rather than the majority of changes that do not affect restriction cleavage sites.

We compared restriction maps among RacL11, Kentucky D and Ab4p. DNA fingerprints of RacL11 and Kentucky D by *Bam*H1 were reported to be varied from that of Ab4p ***(Allen and Bryans, 1986; Hübert et al., 1996; Osterrieder et al., 1996).*** RacL11 and Kentucky D are known to be EHV-1 P genotype ***(Allen and Bryans, 1986).*** However, restriction maps with 8 restriction enzymes used in the present study did not show any genomic differences that could explain the higher pathogenicity of RacL11 and Kentucky D strains. The only difference that we found was a difference in the number of repeat sequences in the intergenic region of ORF62 and ORF63. Restriction maps with 8 restriction enzymes used in the present study did not show any genetic differences that could explain the higher pathogenicity of RacL11 and Kentucky D strains. The EHV-1 modified live-vaccine strain RacH (256th passage on porcine embryonic kidney cells) was investigated by RFLP analysis and compared to representative plaque isolates of the 12th passage (RacL11, RacL22) and 185th passage (RacM24, RacM36) ***(Nugent et al., 2006).*** The restriction patterns of all Rac plaque isolates differed from that of the reference strain Ab4p with missing of *Bam*HI restriction site and 0.1 kbp at the left UL terminus. Losses of restriction

sites without deletions were observed in some Rac derivatives such as RacH, RacM24, RacM36, and RacL22. Identical deletions of 0.85 kbp in size was found in both copies of inverted repeat (IR) regions of RacH ***(Hübert et al., 1996).*** Thus drastic changes such as deletion, inversion and insertion of certain size sequences might not occur as a result of passage in baby hamsters in their genomes. Using LA-RFLP with 8 enzymes which although divide every genomic DNA into 600 fragments seems to be not enough to detect minor variability in genomic sequence and so, more enzymes are required for analysis in future work. DNA genomic restriction sites examined by using eight restriction enzymes were reserved among all studied strains of equine herpesvirus 1 (EHV-1). In conclusion LA-PCR RFLP. However, restriction maps with 8 restriction enzymes used in the present study did not show any genetic differences that could explain the higher pathogenicity of RacL11 and Kentucky D strains. The only difference that I found was a difference in the number of repeat sequences in the intergenic region of ORF62 and ORF63. Using more restriction enzymes for future RFLP comparison is recommended.

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