COMPARISON OF SIX AUSTRALIAN ISOLATES OF BOVINE HERPES VIRUS 2 BASED ON UL24 GENE AFTER A PASSAGE IN MDBK CELLS

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Summary. – A region of the UL24 gene of six Australian field isolates of Bovine herpesvirus 2 (BHV-2) was sequenced after a passage in Madin-Darby bovine kidney (MDBK) cells by polymerase chain reaction (PCR). While the PCR product covered the first half of the UL24 gene, a particular interest was focused on the 274–297 nucleotide (nt) region in which a two nt deletion had previously been detected in the BHM-1 strain of BHV-2. Most isolates tested did not generate any defective UL24 genes during the passage. However, a third of the UL24 genes of BHM-1 strain contained the two nts deletion, but only when a high multiplicity of infection (MOI) was used. Also in the isolate 554 at least a half of the UL24 genes were found to be altered independently of the MOI used. These UL24 genes had an insertion of four nts within the 274–297 nt region. The predicted truncation of the UL24 protein of both viruses occurred at the same stop codon. The region of the gene in which these mutations of the UL24 gene occurred is common to all herpesviruses.

Key words: Bovine herpesvirus 2; Australian isolates; UL24 gene; MDBK cells; mutations

BHV-2 (species *Bovine herpesvirus 2,* genus *Simplexvirus,* subfamily *Alphaherpesvirinae,* family *Herpesviridae*) causes a generalized or teat/udder infections in cattle. An outbreak of bovine mammillitis occurred in 29 herds in late 1973 and early 1974 in a region in Victoria, southern Australia (Turner *et al.*, 1974). While the infection occurred mainly within a 25 km radius it was found also about 110 km away (Turner *et al.,* 1976a,b). The virus isolated from this infection (the isolate 616) and mostly studied further was designated the BHM-1 strain of BHV-2, but also another isolates were obtained from the major infection site at that time (554-Tallygaroopna, 556-Waaia, and 645-Katunga), including one originating from a place 110 km away from that site (566-Koondrook). Another isolate, CSIRO290 originated from Berrimah, Northern Territory, from the blood of a sentinel cow with ringworm-like lesions in 1979 (St George *et al.*, 1980); most of the sentinel cattle and some nearby cattle also showed these (generalized) symptoms of BHV-2 infection at the same time. Generalized (or pseudo-lumpy disease) symptoms of BHV-2 infection are common in tropical conditions (Scott, 1989) but can also be present in the regions where the mammillitis is found (Woods *et al.*, 1996).

While sequencing the genes of BHV-2 (May, 2001), it was observed that the UL24 gene of BHM-1 strain had a two nt deletion within the gene in comparison to strain CSIRO290 (Genebank Acc. No. AY064482), and this was detected in two independently plasmid-cloned UL24 genes in opposite directions. This deletion was not present in the plasmid-cloned UL24 genes of strain CSIRO290 or the genes of its thymidine kinase-deficient derivatives CSIRO290BU3 or CSIRO290BU5 (Genebank Acc. No. AY046323). The complete sequence of BHV-2 strain CSIRO290 UL24 gene indicated that it coded for a protein of 265 amino acids with an estimated M_r of 29,085. At the amino acid level it had 55% homology to Herpes simplex virus 2 (HSV-2), 52%

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Abbreviations: BHV-1 = Bovine herpesvirus 1; BHV-2 = Bovine herpesvirus 2; CHV = Canine herpesvirus; CPE = cytopathic effect; HSV-1 = Herpes simplex virus 1; HSV-2 = Herpes simplex virus 2; EHV-1 = Equine herpesvirus 1; VZV = Varicella-zoster virus; MOI = multiplicity of infection; PCR = polymerase chain reaction

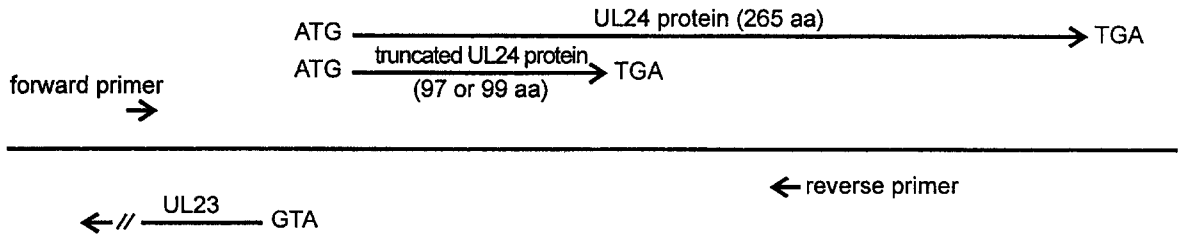

Fig. 1

The effects of alterations to BHV-2 UL24 gene on its protein products

Location of UL24 and UL23 genes, both native and truncated UL24 proteins and primers used in PCR as well as sequencing is indicated.

homology to Herpes simplex virus 1 (HSV-1), 38% homology to BHV-1 and less for most other herpesviruses.

UL24 is considered a core herpesvirus gene, and its gene sequence has been extensively determined for many herpesviruses (Jacobson *et al.*, 1989). Few studies have investigated the protein product of this gene and its role is extensively listed as unknown. Recently the protein encoded by the UL24 gene of HSV-2 has been reported to be a minor capsid protein, possibly a tegument protein (Hong-Yan *et al.*, 2001). The UL24 gene sequence can overlap the UL23 thymidine kinase gene in a head to tail arrangement in most herpesviruses including HSV-1 and HSV-2 (Shimojima *et al.*, 1997), but these genes do not overlap in all herpesviruses (EHV-1 had CHV and VZV; Griffin and Boursnell, 1990). UL24 does not overlap UL23 in BHV-2, there are 74 nts between the genes (Genebank Acc. No. D00537; Sheppard and May, 1989). Studies with HSV-1 UL24, using mutants in the nucleotide sequence not within the UL23 overlapping region, indicate that alterations to the gene can result in smaller plaque size and syncytial plaques, but not always (Jacobson *et al.*, 1989). Furthermore, these HSV-1 UL24 mutants indicated that the gene was important for the infection of mouse sensory ganglia (Jacobson *et al.*, 1998).

To determine whether such UL24 changes actually occurred in BHV-2 strains/isolates, other than BHM-1, the region of the UL24 deletion was sequenced using DNA produced by PCR. MDBK cells were infected with the BHV-2 strains/isolates and when a total cytopathic effect (CPE) became visible total DNAs were extracted from the cells using the Quick-Geno™ Extraction Kit (Clontech) as described previously (Handley *et al.*, 1996). In 1985 we obtained a stock of the original isolate CSIRO290 from Dr T. St. George, Commonwealth Scientific and Industrial Research Organisation, Brisbane, Queensland, Australia, and stocks of five isolates originating from Victoria, namely the Tallygaroopna isolate (11/73; Attwood designated isolate 554), the Waaia isolate (12/73; Attwood designated isolate 556), the Katunga isolate (1/74; Attwood designated isolate 645), and the BHM-1 strain (Attwood designated isolate 616) from the same area, and the Koondrook isolate from Koondrook, 110 km away from the area mentioned above (1/74; Attwood designate 566), all from Dr H. Westbury, Attwood Veterinary Research Institute, Melbourne, Victoria, Australia. A plaque-purifed 554 isolate clone 1 was also included in this study. The infectious titer of stocks of the isolates/strains was 2–5 x 10^6 PFU/ml as titrated on MDBK cells (Handley *et al.*, 1996). The infections for the passage in MDBK cells were carried out both at a low MOI (<1 PFU/cell) and at a high MOI (>5 PFU/cell). DNAs prepared by sodium iodide gradient centrifugation were used in the plasmid-cloning of the UL24 gene (Sheppard and May, 1989; Handley *et al.*, 1996). For the isolate 554 and the 554 clone 1a MOI of less than 0.01 PFU/cell, designated here very low, was also used.

The DNAs extracted from infected cells were used in the PCR performed essentially as described earlier (Handley *et al.*, 1996) but using "a hot start" with the AmpliTaq Gold PCR Master Mixture (Applied Biosystems). Each PCR was done in duplicate. After an initial denaturation and enzyme activation at 95şC for 5 mins 30 cycles of 95şC for 1 min, 55şC for 1 min, and 72şC for 1 min were performed in a thermal cycler (Bresatec). The forward primer 5'-CGCCGTGGTCTTACCCAGTCC-3' starting within the thymidine kinase gene (nt 60–40) and the reverse primer 5'-GAGGATGGGACACAAATACAC-3' starting within the middle of the UL24 gene (nt 447–427) generated a product of 581 bp (Fig. 1). The latter was sequenced at the Monash University Sequencing Facility, Melbourne, Victoria, using the above reverse primer (May, 2001). To detect double sequencing of the region of the UL24 gene exceeding 150 bases due to base deletions or insertions at or near that region of the gene it was essential to evaluate color printouts of the sequencing data.

The sequencing of the PCR products showed that the majority of the virus isolates, namely 556, 645, 566, CSIRO290 uncloned as well as cloned, were identical with the uncloned strain BHM-1, using either low or high MOI (Table 1). However, while the cloned strain BHM-1 (clone 3) showed no variation in the sequence as compared with the uncloned strain BHM-1 at low MOI, it yielded two different sequences occurring after the first 150 nucleotides of the PCR product at high MOI.

The difference resided in two missing nucleotides (tg) in about 30% of the sequences in accord with the sequence of two BHM-1 clones reported previously (Genebank Acc. No. AY064482). Thus for the BHM-1 clone 3 the difference meant an alteration caused exclusively by the high MOI. BHM-1 clone 3 DNA had previously been

Table 1. Nucleotide and amino acid sequences at the start of the conserved region 111 of UL24 gene/protein of some isolates/strains of BHV-2

Virus/strain/isolate tested	Amino acid (upper row) and nucleotide (lower row) sequences at the start of conserved region 111 of UL24 gene/protein											Size of the protein/gene
HSV-1	92	G	G	V	C	V	I	I	E		99	269 aa
	274	ggc	ggg	gtt	tgt	gtc	atc	ata	gaa		297	807 bp
BHV-2: 556, 566, 645, BHM-1	92	G	G	V	C	V	I	V	E		99	265 aa
uncloned, CSIRO290 both uncloned and cloned	274	gga	ggg	gtg	tgt	gtg	ata	gtt	gag		294	795 bp
BHV-2 (tg deleted): BHM-1 clone 3,	92	G	G	V	C	D	S				97	97 aa
high MOI, 30%[a]	274	gga	ggg	gtg	tgt	g--ata	gtt	ga			292	
BHV-2 (aggg inserted): 544 clone 1, very low	92	G	G	R	G	V	C	D	S		99	99 aa
or high MOI, both 80%; 554 clone, any MOI, 50%	274	gga	ggg	agg	ggt	gtg	tgt	gat	agt	tga	300	

[a]The percentage of the altered gene.

produced from cells after a high MOI (> 5 PFU/cell; Sheppard and May, 1989; Maragos *et al.*, 1986) for the determination of the published sequence of strain BHM-1 (Acc. No. AY064482.

During the current sequencing of over twenty complete genes from the BHM-1 strain clone 3 no other similar deletion has thus far been detected in any other gene. However, the 554 isolate was found to produce an altered UL24 gene, whether inoculated at a very low (0.01 PFU/cell), low or high MOI. The altered DNA appeared in about 50% of the DNA sequences and in this case, in contrast to the strain BHM-1 clone 3, the MOI had no influence on the amount of the altered DNA produced.

Furthermore, the sequence in all PCR products from the isolate 554 had an insertion of four nucleotides (aggg). Even after plaque purification, the isolate 554 clone 1,when tested either at a very low or high MOI, yielded a product in which 80% of the DNA contained the insert of four nucleotides (aggg) in the gene. Thus the isolate 554 produced an altered UL24 gene sequence during the passage independently of the MOI used. Table 1 indicates the changes detected within the UL24 gene of the strain BHM-1 clone 3, the isolate 554 clone 1 as well as uncloned respective viruses. While the alterations to the UL24 gene are different (deletion and insertion) in the two BHV-2 strains BHM-1 and 554 they both terminate the UL24 protein at virtually the same region (Fig. 1). The changes occurred in the region 111 conserved in all herpesviruses (for HSV-1 see Table 1; Jacobson *et al.*, 1989,1998). The strain BHM-1 clone 3 and the isolate 554 are predicted to code for a truncated 97 and 99 amino acid protein, respectively. At no stage was the altered UL24 gene detected exclusively in the PCR products, it was always present along with some (20-70%) unalterd DNA (Table 1).

Little is known about the UL24 protein or its role in herpesvirus replication. Recently it has been suggested that it is a minor capsid protein present in all herpesviruses and having some specific role (Hong-Yan *et al.*, 2001). The significance of the fact that the UL24 gene is altered in some strains/isolates of BHV-2 during their passaging in a MDBK cell culture remains unknown. The alteration is related to the MOI in the strain BHM-1 clone 3 as demonstrated here; a high MOI is associated with the generation of defective particles of many viruses, but not necessarily of all herpesviruses. However, this is not the case of the isolate 554. It is possible that its DNA containing an altered UL24 gene can be replicated along with the normal (unaltered) DNA, similarly to the situation with some HSV-1 mutants (Jacobson *et al.*, 1989, 1998); if not, such an alteration is certainly readily generated in the isolate 554 even after plaque purification.

The outbreak of mammillitis in Victoria, Australia during 1973/1974 has never reoccurred and no infection has subsequently reappeared. One study has suggested that the UL24 gene/protein is essential for the initiation of latency (Jacobson *et al.*, 1998). As the strain BHM-1 was initially thought to have an inactivated UL24 gene, this study was undertaken to determine if all of the five isolates from the outbreak also had an inactive UL24 gene. However, none of the original isolates tested produced a totally defective UL24 gene, four strains even never generating a defective UL24 gene during a passage in a cell culture, suggesting that a lack of an active UL24 gene was not the cause of the virus failure to become latent and persisting after the outbreak.

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