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Isolation of a naturally occurring vaccine/wild-type recombinant bovine herpesvirus type 1 (BoHV-1) from an aborted bovine fetus

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ABSTRACT

Bovine herpesvirus type 1 (BoHV-1) causes various disease syndromes in cattle including respiratory disease and abortions. During an investigation into the potential role of BoHV-1 modified-live vaccines (MLV) causing diseases in cattle, we performed whole genome sequencing on six BoHV-1 field strains isolated at Cornell Animal Health Diagnostic Center in the late 1970s. Three isolates (two respiratory and a fetal) were identified as vaccine-derived isolates, having SNP patterns identical to that of a previously sequenced MLV virus that exhibited a deleted US2 and truncated US1.67 genes. Two other isolates (a respiratory and a fetal) were categorized as wild-type (WT) viruses based on their unique SNP pattern that is distinct from MLV viruses. The sixth isolate from an aborted fetus was a recombinant virus with 62% of its genome exhibiting SNPs identical to one of the above-mentioned WT viruses also recovered from an aborted fetus. The remaining 38% consisted of two blocks of sequences derived from the MLV virus. The first block replaced the UL9-UL19 region, and the second vaccine-derived sequence block encompassed all the genes within the unique short region and the internal/terminal repeats containing the regulatory genes BICP4 and BICP22. This is confirmatory evidence that recombination between BoHV-1 MLV and WT viruses can occur under natural conditions and cause disease. It is important in that it underscores the potential for the glycoprotein E negative (gE$^-$) marker vaccine used to eradicate BoHV-1 in some countries, to recombine with virulent field strains allowing them to capture the gE$^-$ marker, thereby endangering the control and eradication programs.

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1. Introduction

Bovine herpesvirus type 1 (BoHV-1) is an alphaherpesvirus that causes significant disease in cattle including respiratory, reproductive tract, fetal, and neonatal infections [9,12,18]. BoHV-1 belongs to the order *Herpesvirales* and has been placed in the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus*. There are three subtypes of BoHV-1: BoHV-1.1, also referred to as the respiratory strain, and two genital strains BoHV-1.2a and BoHV-1.2b [6,18]. To date, the complete genome sequences of nine BoHV-1 modified-live vaccine (MLV) viruses from commercially available vaccines have been determined as well as the complete genome sequences of approximately 40 BoHV-1 field isolates from bovine respiratory disease, genital tract infections, aborted fetuses, and systemic infections in neonatal calves [6,10–12]. The MLV viruses fell into 4 vaccine groups based on their single nucleotide polymorphism (SNP) patterns [11,12]. The non-vaccine associated wild-type (WT) viruses all fell into a separate group having significantly different SNP patterns to those of the MLV viruses. All WT BoHV-1 viruses sequenced to date share a number of very distinct SNPs, none of which are present in any of the MLV viruses sequenced [10–12].

Although BoHV-1 MLV vaccines provide protection against disease in adults [2], they can also cause disease. For example, MLV virus from each of the four vaccine groups have been isolated from cattle with respiratory disease, aborted bovine fetuses, and/or neonatal calves with systemic infections [10–12]. It is interesting that outbreaks of postvaccinal BoHV-1 abortion have been reported with increased frequency since the early 2000s, coinciding with the licensure in 2003 of MLVs intended for use in pregnant cattle [23]. It is not certain if these MLV viruses can cause disease *per se*, or if their ability to cause disease is as a result of recombination with more virulent WT viruses. BoHV-1 MLV viruses are

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administered to cattle upon entering feedlots at a time when WT viruses may also be circulating in these animals. Co-infection of cattle with both the MLV and WT viruses provides the necessary situation for generation of more virulent vaccine/WT recombinant viruses. However, full genome sequencing of numerous BoHV-1 vaccine-derived strains isolated from diseased cattle has not revealed any obvious recombinants to date, although some vaccine-derived isolates do have a few additional SNPs and/or insertions and deletions (indels) as compared to the MLV virus from which they are derived [10–12].

Homologous recombination between strains of the same alphaherpesvirus species have been reported to occur frequently, both in vitro and in vivo, between strains of herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), pseudorabies virus, feline herpesvirus 1, equine herpesvirus type 1 (EHV-1), and BoHV-1 [8,13,14,21,26,29]. Recombinants have also been reported between alphaherpesviruses of different but closely related species, e.g. EHV-1 and EHV-4 [24] and BoHV-1 and BoHV-5 [3,17]. Recombination among strains of live-attenuated Gallid herpesvirus 1, which was first reported in Australia in 2008, continues to emerge on that continent [1,15]. Intranasal co-inoculation of cattle with two distinct BoHV-1 mutants generated multiple recombinants which were isolated both during the primary infection and after reactivation from latency [26]. However, simultaneous or closely separated infections (maximum four hours) with a high dose of two viruses was required to generate recombinant viruses [29]. Consequently, it has been suggested that the likelihood of such simultaneous events occurring in cattle under natural conditions (cellular coinfections with high doses of two virus within a four-hour time period) and generating recombinants would be a rare event [29].

There are no reports to date of isolation of either a field isolate of BoHV-1 generated by the recombination between WT viruses, or a recombinant between an MLV and a WT virus. In this report, we describe the isolation of a naturally occurring MLV/WT double recombinant of BoHV-1 isolated from an aborted bovine fetus. The recombinant was discovered during the analysis of six BoHV-1 strains isolated in 1978 and 1979 at the Animal Diagnostic Center at Cornell, NY. The nucleotide sequence and genetic differences among the genomes are described, along with a description of the genome of the recombinant and the source of MLV and WT sequences that make up the recombinant virus.

2. Materials and methods

The BoHV-1 strains sequenced were isolated at the Animal Health Diagnostic Center, Cornell University, Ithaca, NY. Isolates were from calves with respiratory disease or from aborted bovine fetuses. We selected six of the oldest isolates for whole genome sequencing. Table 1 lists the virus isolates sequenced, their GenBank Accession numbers, dates of submission, and a brief history of each sample.

All isolates were propagated on Madin Darby bovine kidney (MDBK) cells before viral DNA was extracted and sequenced using a combination of Illumina and Sanger sequencing as described [4–6,10]. The CLC Genomics and Main Workbench programs (CLC bio, Germany) were used for sequence analysis as previously described [6]. Both *de novo* assembly of reads, and assembly of reads to the reference BoHV-1 Cooper strain genome [GenBank Accession JX898220] were performed. Gaps in the genome sequence were filled by examining individual mapped and unmapped reads. Because of the inability of the automatic alignment algorithms to resolve the exact number of repeats in some regions of the genome containing multiple reiterated tandem-repeats, Sanger sequencing was performed on PCR-amplified fragments within the reiterated repeat regions of the UL36 and BICP4 genes, and the intron spliced out of the immediate early RNA (IER1.7) gene that encodes BICP22 [27].

Table 1
List of virus isolates and MLV viruses sequenced in this study, along with their identification numbers, date of submission, a short history, and GenBank Accession numbers.

Reference/case	Virus identification	History of virus isolate	GenBank Accession Number
Cooper	IBR Cooper strain	USDA NVSL challenge 97-11	JX898220
V1[a]	Ser# 165-004	Commercially available modified-live (MLV) Arsenal IBR virus vaccine	MH724202
V9	Nasalgen MLV intranasal vaccine	Obtained from Jensen-Salsbery Laboratories, Kansas City, MO.	MH724210
C42	A76729	Sample submitted 01/03/1978; isolated from tissue obtained from an aborted fetus	MH791336
C43	A78878	Sample submitted 01/30/1978; isolated from nasal wash from calf with respiratory disease	MH791337
C44	A78911	Sample submitted 01/30/1978; isolated from nasal wash from calf with respiratory disease	MH791338
C45	A147431	Sample submitted 12/18/1979; isolated from nasal wash of a calf with respiratory disease	MH791339
C46	A141493	Sample submitted 10/22/1979; isolated from tissue obtained from an aborted fetus	MH791340
C47	A76806	Sample submitted 01/03/1978; isolated from tissue obtained from an aborted fetus	MH791341

[a] The SNP pattern of this virus has been previously described [9,10].

The latest version of the Recombinant Detection Program RDP4 was used to detect and visualize the recombination events and to determine approximate recombination points [16]. Five different recombination tests were conducted using the following programs: GENECONV, Chimaera, MaxChi, BootScan, and 3Seq.

The GenBank Accession numbers detailing the complete genome sequences of the isolates and vaccines strains used for comparison purposes are listed in Table 1.

3. Results

3.1. Sequencing and coverage

The Illumina sequencing data were obtained from an average of 10.9×10^6 matched-reads per sample (range 10.3×10^6–12.4×10^6) which covered the reference Cooper genome (GenBank Accession JX898220) at an average depth of 18,930-fold per sample (range 15,640–21,280). The mean length of reads ranged from 251 to 266 bp. At a 30-fold coverage, the probability of a correct SNP being called is estimated at 99% (Illumina Technical Notes). To maximize confidence in calling SNPs in this study, the minimum coverage required to call a SNP was set at 35-fold coverage. Read depth coverage in the unique long (UL) and unique short (US) regions of the genome was consistently high except for UL36 which contains a 1.7 Kb region consisting of eight different reiterated repeat regions that each contain multiple tandem repeats. The internal repeat (IR) segment of the genome between the UL and US

regions (and its duplicated inverted terminal repeat (TR) segment) had areas of low coverage due to the presence of numerous reiterated repeat regions. The number of repeats in these areas of low coverage were resolved both by examining individual mapped and unmapped reads, and by performing Sanger sequencing of PCR-amplified products that spanned these reiterated repeat regions. Similar problems involving low coverage within regions of the genome containing multiple copies of tandem repeats, and of the inability of the automatic alignment programs to resolve all the repeats in such regions, has been reported during high-throughput Illumina sequencing of HSV-2 [20].

3.2. SNP detection and comparison of genomes

The isolates sequenced and listed in Table 1 were either isolated from nasal washes of calves with respiratory disease (C43, C44, C45) or from tissue of aborted fetuses (C42, C46 C47). Table 2 lists the SNP patterns for the two WT isolates (C42 & C43), a vaccine-derived isolate (C44), the recombinant virus (C47), as well as the commercially available vaccine V9 sequenced in this study (GenBank Accession MH724202). The SNP patterns of the two other vaccine-derived isolates (C45 & C46) are not shown since they were identical to that of MLV virus V9. Fig. 1 graphically shows and compares the location of individual SNPs detected in the WT virus C42, the vaccine virus C44, and the recombinant virus C47, when compared to the reference Cooper strain sequence JX898220.

As observed in Table 2, the MLV virus V9 and vaccine-derived isolate C44 have identical SNP patterns except for a mutation at position 41,024 of the reference Cooper strain sequence. Furthermore, that "vaccine" SNP pattern is noticeably different and distinct from those of the two WT isolates C42 and C43. Although these WT isolates share a number of SNPs (many of which are

Table 2
Summary of single nucleotide polymorphisms (SNPs) detected in the genomes of BoHV-1 wild-type viruses C42 and C43, MLV virus V9, vaccine-derived isolate C44, and recombinant virus C47.

BoHV-1 Genome GenBank JX898220[a]			Vaccine and Virus Isolates				
Genes	Position[b]	Base	V9	C44	C47	C42	C43
UL54	*2770*	T	C[c]	C	·[d]	·	·
UL53	*3466*	T	·	·	C	C	C*
UL52	*4560*	C	·	·	T	T	T*
UL52	*4848*	C	·	·	·	·	T
UL52	*5282*	G	·	·	T	T	T*
UL52	*5585*	C	·	·	T	T	T
UL52	*6096*	A	·	·	·	·	G
UL50	*9034*	G	·	·	·	·	A
UL49	*10096*	G	·	·	·	·	A
UL48	*11591*	G	·	·	A	A	A*
UL47	*12360*	C	T	T	·	·	·
Intergenic	*14295*	A	·	·	G	G	G*
Intergenic	*14298*	A	·	·	G	G	G*
UL46	*14409*	G	·	·	A	A	A*
UL46	*15980*	T	·	·	C	C	·
UL46	*16013*	T	·	·	C	C	·
UL46	*16265*	C	·	·	·	·	T
UL46	*16536*	T	C	C	·	·	·
UL44	*17821*	T	C	C	·	·	·
UL44	*17826*	T	C	C	·	·	·
UL43	*18913*	C	T	T	·	·	·
UL42	*20049*	C	·	·	·	·	T
UL41	*21814*	G	·	·	A	A	A
UL39	*23908*	C	·	·	G	G	G*
UL39	*25768*	C	·	·	T	T	T*
UL38	*26698*	T	C	C	·	·	·
UL37	*30283*	C	T	T	·	·	·
UL36	*32339*	C	·	·	T	T	T*
UL36	*35423*	G	A	A	·	·	·
UL36	*35934*	A	G	G	·	·	·
UL36	*37621*	T	C	C	·	·	·
UL36	*37788*	A	G	G	·	·	·
UL35	*41024*	T	·	G	·	·	·
Intergenic	*42183*	G	·	·	·	·	A
UL30	*46433*	C	·	·	T	T	T
UL30	*46727*	G	·	·	·	·	A
UL30	*47124*	C	·	·	T	T	·
UL30	*47931*	G	·	·	·	·	A
UL29	*50693*	C	T	T	·	·	·
UL28	*55156*	G	·	·	C	C	·
UL27	*55842*	C	·	·	A	A	·
Intergenic	*58807*	T	C	C	·	·	·
Intergenic	*58810*	C	·	·	·	·	T
UL23	*64034*	G	·	·	·	·	A
Intergenic	*67293*	T	·	·	·	·	G
UL21	*67868*	G	T	T	·	·	·
UL21	*68148*	G	·	·	T	T	T
Intergenic	*69914*	A	·	·	G	G	G*
UL19	*70324*	T	C	C	C	·	·
UL19	*70328*	C	·	·	·	·	T
UL18	*75117*	C	T	T	T	·	·
UL15	*75597*	G	·	·	·	·	A
UL13	*82423*	A	·	·	·	G	G*
UL13	*82547*	A	·	·	·	G	G*
UL13	*82894*	C	·	·	·	T	T*
UL8	*88971*	G	·	·	·	A	·
UL5	*94599*	C	·	·	T	T	T*
UL5	*95871*	T	·	·	C	C	C*
Intergenic	*98173*	G	·	·	A	A	A*
Intergenic	*98175*	G	·	·	C	C	C*
Intergenic	*98177*	T	·	·	C	C	C*
UL0.5	*100236*	C	·	·	T	T	T*
UL0.5	*100249*	T	C	C	·	·	·
BICP0	*102452*	C	T	T	·	·	·
Intergenic	*103766*	C	·	·	·	·	T
Intergenic	*110920*	T	·	·	·	·	C
Intergenic	*111747*	A	·	·	·	G	·
Intergenic	*111910*	C	·	T	T	·	·
Intergenic	*112514*	C	·	·	·	T	T
Intergenic	*114067*	G	·	·	·	·	A
US3	*116042*	A	·	·	·	G	G
US3	*116043*	A	·	·	·	G	G*
US3	*116045*	A	·	·	·	G	G*
US3	*116632*	C	·	·	·	T	·
US4	*117521*	G	·	·	·	·	A
US7	*120936*	A	·	·	·	G	G*
Intergenic	*121521*	G	·	·	·	A	·
US8	*122021*	C	·	·	·	·	A
US8	*122227*	A	G	G	G	·	·

[a]The reference genomic map is based on the complete BoHV-1.1 Cooper genome (GenBank Accesion JX898220).
[b]Nucleotide position on the NVSL BoHV-1 Cooper reference genome.
[c]SNPs: the probability of any one of the above SNPs being called is estimated at 99% (Illumina Technical Notes).
[d]Dots in the Table represent nucleotides that match those in the reference genome.
*Nucleotides with an asterisk denotes SNPs that are present in all BoHV-1 wild-type (WT) viruses sequenced to date.
Boxed areas represent regions of the genome where SNPs of recombinant virus C47 has been replaced by SNPs of the vaccine virus C44.

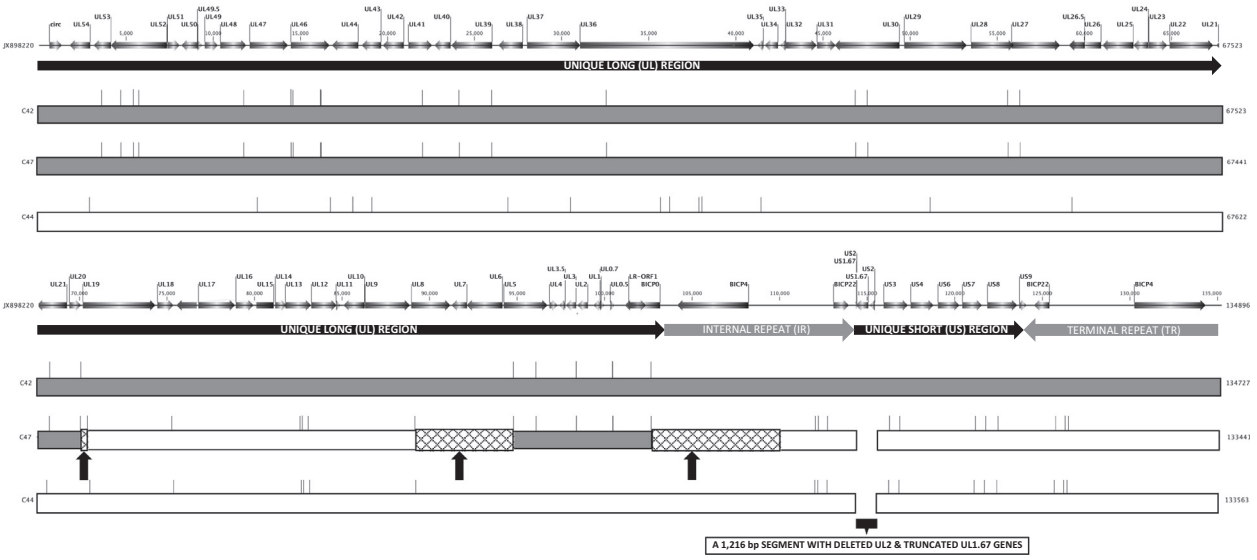

Fig. 1. Graphical representation of the genomes of three (of six) 1978/1979 BoHV-1 strains isolated and sequenced in this study. C42 is a WT virus isolated from an aborted bovine fetus, C44 is a vaccine-derived respiratory isolate, and C47 is a double recombinant virus isolated from an aborted fetus. The annotated reference Cooper strain genome (GenBank JX898220) is shown at the top. The vertical bars on the genomes represent the location of SNPs described in Table 2. The change in SNP pattern and color fill in the recombinant C47 genome represent two regions of the genome where the vaccine sequences (white-colored genome) have been inserted into the WT genome (dark-colored genome) during recombination. The crosshatched areas on the recombinant C47 genome represents regions of the genome where the crossover from the MLV to WT sequence occurred. The vertical dark arrows represent the most likely location within these crossover regions where the crossover event (breakpoint) occurred, as calculated by the RDP4 Recombination Detection Program. The 1216 bp sequence of the C44 and C47 genomes containing the deleted US2 and truncated US1.67 genes are shown.

denoted by an asterisk in Table 2), they possess enough additional SNPs to easily distinguishable one from the another. Thus, the SNPs presented in Table 2 clearly demonstrates that the SNPs pattern of the recombinant virus C47 is more closely related to that of the WT virus C42 than that of the WT virus C43.

3.3. Wild-type BoHV-1.1 isolates

Of the two WT isolates identified in this study (C42 & C43), one was isolated from aborted fetal tissue and the other was a respiratory isolate. While they share many of the same SNPs, 23 of which are identified by an asterisk in Table 2, these two WT isolates can easily be distinguished from each other by the fact that the respiratory isolate (C43) has 19 additional SNPs not detected in the fetal isolate (Table 2). These 23 above-mentioned shared SNPs have been described in all non-vaccine associated BoHV-1 WTs but not in any MLV viruses described to date [10,11].

3.4. The modified-live vaccine virus isolates

Except for one SNP in C44 at position 41,024 of the reference Cooper strain sequence (Table 2), the SNP patterns of the three vaccine-derived isolates (C44, C45, C46) were identical to those described for the commercially available MLV viruses V1 [10] and V9 that was sequenced as part of this study and shown in Table 2. Both V1 and V9 are classified under the Group 1 vaccines [10]. Furthermore, the genomes of these two MLV viruses, as well those of the three vaccine-derived isolates (C44, C45, C46), are similarly unique in that they all have a deleted US2 gene and a truncated US1.67 gene which lies upstream of the deleted US2 gene (Fig. 1). Despite these genetic similarities, however, the two MLV viruses and three vaccine-derived isolates can be individually distinguished through the presence of indels not shown in Table 2. Most of these indels result from differences in the number of copies of tandem repeat units within reiterated repeats regions of the genome, especially within UL36 and the intron excised from IER1.7 just upstream of BICP22. It is these indels that account for the different lengths of their genomes.

3.5. Recombinant virus isolate C47

As Table 2 indicates, the isolate C47 has a very similar SNP pattern to that of the WT isolate C42. In addition, C42 and C47 share four SNPs (at positions 15980, 16013, 47124, 55842; Table 2) that are absent from the WT C43 genome. However, there are two regions of C47 genome that do not share SNPs with WT isolate C42, but rather exhibit SNPs identical to the vaccine-derived isolates (boxed areas in Table 2). Thus, isolate C47 appears to be a recombinant virus probably derived from the WT isolate related to isolate C42 (also isolated from an aborted fetus) and an MLV derived virus like isolate C44.

As shown in Fig. 1 and Table 2, the recombinant C47 and WT C42 have identical SNPs up to position 69,914 which is located in the non-coding intergenic region between the UL19 and UL20 genes, while the next SNP located 410 bp further along the C47 genome (position 70,324) matches that of the vaccine-derived isolate C44. Thus, a crossover event has occurred within this 410 bp area (between positions 69,914 & 70,324). Comparing C47 and C44/C42 SNPs, a second crossover point occurs within a 5628 bp region spanning the UL5-UL8 genes (positions 88,971 & 94,599). The next 7853 bp stretch of the recombinant genome is clearly derived from of the C42 WT virus (positions 94,599–102,452). A third recombination event occurs within an 8408 bp span beginning in the BICP0 coding sequence and ending just downstream of BICP4 within the internal/terminal repeat (IR/TR) region (position 102,452–110,044), coinciding with the start of a few indels shared by C44 and C47. From this point on, the entire C47 genome SNP pattern corresponds to that of the vaccine-derived C44 isolate. Thus, the entire US region with the deleted US2 and truncated US1.67 is derived from the vaccine virus. Depending on where the breakpoint is located within the crossover region (positions 102,452–110,044), either (1) the entire IR/TR region of the vaccine virus, including BICP4, crossed over to the WT genome, or (2) only

part of the IR/TR regions crossed over, with the genome retaining part or all the WT BICP4 gene (Fig. 1).

Although the distinctive SNPs present in WT and vaccine genomes allowed us to identify the location of the heterologous sequence and crossover regions by visual examination of the aligned genomes (Fig. 1), a recombination detection program was used to confirm these observations and to determine more precisely the location of the breakpoints within the crossover regions. All recombination tests conducted using the RDP4 program detected two recombination events in which the WT virus received two blocks of sequences from the vaccine virus. The program calculated the location, where the breakpoints had the highest probability of occurring, at positions 70130, 91792, and 104,494 as shown in Fig. 1. Because of possible inaccuracies in the predicted breakpoint positions [16; Instruction Manual], we are still unsure of the exact location of the breakpoint in the IR/TR region of the recombinant genome.

In summary, the genome of the isolated double recombinant C47 is 133,441 bp long and made-up primarily of WT sequences totaling 62% of the genome. Approximately 80% of the UL region was WT virus sequence, into which was inserted a minimum of 18,647 bp of vaccine virus sequence. Most, if not all the entire 29,675 bp recombination site consisting of the IR, US, and TR regions of the genome was primarily derived from the vaccine virus.

4. Discussion

As part of an investigation into the evolution of BoHV-1, we sequenced the entire genome of six BoHV-1 strains isolated ~40 years ago and archived at the Animal Health Diagnostic Center, Cornell University, Ithaca, NY. Half of the isolates were recovered from nasal secretions of cattle with respiratory disease and the other half from aborted fetal tissue. Four of the isolates were initially identified as vaccine-derived viruses by their deleted US2 gene and a truncated US1.67 gene as depicted in Fig. 1. The other two isolates were categorized as WT viruses based upon their SNP patterns. However, upon close examination of one of the isolates initially identified as a "vaccine" virus with the US2/US1.67 deletion, it was discovered that the SNPs in the UL region matched those of the WT, with the exception of one small sequence block with SNPs that matched those of the vaccine viruses. This isolate (C47) was thus revealed to be a recombinant virus, having distinct regions of its genome derived from both a vaccine and WT virus.

Two of the six isolates in this study (C42, C43) were classified as WT based on the fact that they share 23 unique SNPs (denoted by asterisks in Table 2) that have been recorded only in the genomes of non-vaccine associated WT viruses described, [10,11] and/or sequenced to date (GenBank Accession Numbers: MK552112, MH598936-MH598938). Despite their shared SNPs, however, they can be easily distinguished by the fact that the genome of respiratory isolate displays many additional SNPs not observed in that of the fetal isolate C42. It is noteworthy that C42 represents the first WT virus isolated from a naturally infected aborted fetus in the US; heretofore, all naturally occurring field isolates of BoHV-1 from aborted fetuses that have been genetically analyzed to date are related to vaccine strains [2,10–12,23].

Although the three vaccine-derived isolates have almost identical SNP patterns to those of the two MLV viruses V1 and V9, it is more likely that the vaccine isolates C44, C45 & C46 were derived from V9. The MLV vaccine V9 was originally marketed in the 1970's as an intranasal vaccine by Jensen-Salsbery Laboratories as Nasalgen 1 (Table 1), but through a series of mergers/acquisitions, it is now marketed as Nasalgen® IP (Merck Animal Health, Madison, NJ). The fact that the MLV vaccine V1 is marketed as a vaccine given parenterally, and that vaccine-derived viruses C44 and C45 were recovered from calves with respiratory disease, further supports the probability that the 1978/1979 vaccine-derived isolates sequenced in this study were derived from V9. Although Group 2, 3 and 4 vaccine viruses have all been isolated from cattle with bovine respiratory disease [12], this is the first report of a Group 1 vaccine virus isolate from cattle with respiratory disease (isolates C44 & C45), and the second recorded isolate from an aborted fetus (C46). The previous isolations of a Group 1 virus (V1) were from the adrenal tissue of a neonatal calf with systemic BoHV-1 infection [10] and from an aborted bovine fetus [2].

All in vivo BoHV-1 experimental recombinants generated have been through co-inoculation of cattle with two different mutants of BoHV-1 intranasally, the natural route for BoHV-1 infection [26]. Recombinant viruses were not only generated during primary infection in the nasal mucosa, but could be re-isolated in the nasal secretions during recrudescence after a period of latency [26,29]. Unlike the above study, the recombinant in this study was isolated from an aborted bovine fetus which is not directly connected to the site of replication where vaccines are administered and recombinants would be expected to develop. Therefore, our recombinant would have had to gain access to the placenta/fetus via the hematogenous route following its presumed generation in the respiratory tract.

Hematogenous spread of BoHV-1 to the fetus is thought to occur via cell-associated viremia in infected monocytes/lymphocytes [22]. These blood cells can become infected during replication of BoHV-1 in the nasal mucosa, tonsils, and other respiratory-associated mucosal tissue [31]. Therefore, it is possible for lymphocytes/monocytes to become infected with a recombinant generated in the nasal passages and associated lymphoid tissues, and to carry the recombinant to the placenta/fetus where it can replicate resulting in abortion. In the case of EHV-1, it has been clearly demonstrated that T-cells become infected through contact with infected respiratory epithelial cells, after which the infected T-cells carry the virus to the endothelium cells of target organs such as the uterus where the virus replicates [25]. Recombinants between a vaccine and WT virus could be generated intranasally during the simultaneous infection of cattle with a vaccine and WT virus, as might occur in cattle vaccinated intranasally during an outbreak of bovine respiratory disease with a WT virus [29]. Alternatively, recombinants could be generated in the nasal passages of an animal that was (1) previously infected with a WT virus and vaccinated intranasally during recrudescence of the latent WT virus, or (2) latently infected with both WT and vaccine virus that replicated after recrudescence of both viruses. It has been shown that cattle can be latently infected with two distinguishable strains of BoHV-1 [30], and that a single neuron can be dually infected with two different alphaherpesviruses, such as HSV-1 and VZV [28].

Although the above-mentioned scenarios are the most plausible explanation for the generation and hematogenous spread of a vaccine/WT recombinant virus to the placenta/fetus, it is also possible that the recombination occurred within circulating lymphocytes/monocytes dually infected with a vaccine and WT viruses. The simultaneous presence of WT and a gE$^-$ BoHV-1 marker vaccine virus has been detected in circulating peripheral blood leucocytes (PBL) of cattle vaccinated with the gE$^-$ mutant [7]. Moreover, this gE$^-$ vaccine virus could be detected in the PBLs of vaccinated cattle as long as five months post vaccination. The fact that we and others have recovered and described vaccine-derived virus associated with abortions in herds where no vaccines had been used for an extended period of time (over two years in one case) demonstrates the ability of BoHV-1 to remain latent in tissues, which raises the possibility of latently infected cells becoming dually infected with different stains of BoHV-1 [2,12].

The underlying mechanisms to explain recombination between alphaherpesviruses are poorly understood. However,

recombination is associated with DNA replication and is a mechanism used not only for repair of DNA damage, but also to exchange genetic segments between different strains of viruses [21,29]. The recombination event observed in the UL region of the double C47 recombinant recovered in this study could be explained by double-strand break repair model, the current model of homologous recombination that might occur during herpesvirus replication in a cell dually infected with two genetically related alphaherpesviruses [29]. On the other hand, the recombination event involving the switching of the wild-type IR/US/TR segment of the genome by the vaccine IR/US/TR sequence, is likely due to the presence of inverted repeats inside the alphaherpesvirus genome that has been shown to be involved in intramolecular recombination [29]. When tissue culture cells or animals are infected simultaneously with two strains or species of BoHV-1 that can be distinguished by markers in both the UL and US regions, recombinants are generated with the UL region of one mutant and US region of the other [3,19,26,29]. Therefore, such a natural recombination event may well explain the emergence of our naturally isolated vaccine/WT recombinant in which the IR/TR region of the WT genome has been replaced with either part, or all of the IR/TR region originating from the vaccine virus.

Of the six virus isolates sequenced in this study, the WT virus C42 and the recombinant virus C47 were both isolated from different aborted fetuses necropsied on the same date (Table 1). This is noteworthy, in view of the fact that the WT sequence in the recombinant virus C47 was derived from a WT virus that was genetically identical to C42. The isolation of three isolates derived from the same vaccine V9 (C44 in 1978, C45 & C46 in 1979) is good evidence that cattle were being vaccinated with vaccine V9 in the same general geographical area. Unfortunately, apart from what is shown in Table 1, no additional history was available regarding the source of the animals or fetuses from which the six virus strains were isolated. However, four viruses were isolated in the same month: the WT viruses C42&C43, vaccine-derived virus C44, and the recombinant virus C47 (Table 1). Had all these viruses been recovered from neighboring farms, it would be strong evidence that vaccination had occurred at around the same time as the abortions occurred, which could help explain the origin of our MLV/WT recombinant isolated in this study. It has been proposed that vaccination of cattle with the attenuated gE$^-$ intranasal marker vaccine during an outbreak of respiratory disease with a virulent field strains BoHV-1, could be an event which would favor the generation of recombination between a BoHV-1 field strain and the gE$^-$ vaccine virus used in some countries for the eradication of BoHV-1 [7,29]. The isolation of a natural vaccine/WT recombinant during this study, in which the entire US region was recombined, highlights the fact that such recombinants could be generated, with a virulent WT acquiring the gE$^-$ marker, or the gE$^-$ vaccine acquiring the wild-type gE, thus endangering the control and eradication programs.

5. Conclusions

In this study, we sequenced and analyzed the complete genome sequences of six BoHV-1 strains isolated in 1978/1979 at the Animal Diagnostic Center at Cornell, NY. Half were isolated from the respiratory tract of cattle with respiratory disease, and the others from aborted bovine fetuses. Both WT and vaccine-derived BoHV-1 were isolated from the samples. However, one isolate recovered from an aborted fetus was discovered to be a naturally occurring recombinant between the vaccine and the WT viruses. Based on SNP patterns, most of the recombinant virus genome was derived from a WT virus, with two blocks of sequences derived from a vaccine strain. This is confirmatory evidence that recombination between BoHV-1 MLV and WT viruses can occur under natural conditions and cause disease. We also confirm, by whole genome sequencing, the isolation of a WT BoHV-1 from an aborted fetus; heretofore, all abortion isolates in the US had been uniformly vaccine derived. The result of this study underscores the potential for gE$^-$ marker vaccine, used in the eradication of BoHV-1 in some countries, to recombine with virulent field strains, thereby endangering the control and eradication programs.

Declaration of Competing Interest

None of the authors have any personal or financial conflict of interest that would bias the results of this study.

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