SEQUENCING FIELD ISOLATES OF ILTV

by

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ABSTRACT

The objective of this project is to develop new sequencing technology which will be used to determine and analyze the infectious laryngotracheitis virus (ILTV) genome. Infectious laryngotracheitis is an acute respiratory tract infection of birds. The disease spreads rapidly and is characterized by bloody mucus, gasping, and high mortality. The virus itself is classified as an alpha herpesvirus (Gallid herpesvirus 1), with a genome approximately 150,000 nucleotides in length (49).

By using next-generation sequencing (Illumina) technology we have sequenced two field isolates of ILTV. The first isolate (1874C5) is a 2005 field isolate from Georgia. The second isolate (632) is a 1985 Delaware field isolate. The sequences of these two isolates were compared to show how similar the strains remain across time and geographical location.

The results of sequencing for the 1874C5 strain yielded a total of 6,122,687 ILTV bases that aligned to the published reference sequence (49). For the 632 strain, a total of 432,786,937 ILTV bases were sequenced which aligned to the reference. Analysis of the 632 and 1874C5 isolates show that the two sequences are 97.6% similar with only 128 single nucleotide polymorphisms (SNPs) between them, and that the Delaware isolate contains a 3,333 base pair deletion from nucleotide 2173 to 5746.

Chapter 1

INTRODUCTION

1.1. Infectious Laryngotracheitis (ILT)

While infectious laryngotracheitis tends to stay out of the media spotlight, it is a concern for poultry farmers and countries with intensive poultry production or large concentrations of poultry such as the US, Europe, China, Southeast Asia, and Australia. The United States Poultry industry reports that ILT is responsible for multimillion dollar losses each year in the production of both eggs and chicken (45).

The disease exhibits a variable mortality rate (5-70%) but generally 10-20% mortality is observed (1, 23, 48). However, the major economic impact of the disease is that it is highly contagious (90-100% morbidity) and will decrease rates of growth in broiler chickens and egg production in hens (1, 23, 48). Clinical signs of ILT generally appear 6-12 days following natural exposure (31, 48), but with experimental intratracheal inoculation the incubation period can be reduced to only 2-4 days (3, 28, 48). Unfortunately for producers and veterinarians, the clinical symptoms associated with mild cases of ILT may be difficult to distinguish from those of other acute respiratory diseases and thus the disease can be difficult to diagnose. Common symptoms associated with the disease include: nasal discharge, moist rales, watery eyes, conjunctivitis, swelling of the infraorbital sinuses, mild tracheitis, coughing, gasping, decreased egg production, and general unthriftiness (1, 31). The diagnosis of ILT often requires the aid of a laboratory to distinguish between avian poxvirus, Newcastle disease virus, avian influenza virus,

infectious bronchitis virus, fowl adenovirus, and Aspergillus. Possible laboratory tests include: histopathology (intranuclear inclusion bodies), methods for detecting viral antigens (FA, IP, enzyme-linked immunosorbent assays--ELISA), and PCR or DNA hybridization techniques (45). Only severe acute forms of ILT which cause dyspnea, expectoration of blood-stained mucus, and high mortality can be reliably diagnosed on the basis of clinical signs (1, 23, 24, 29, 48). The intensity of the disease varies with the severity of lesions, but most chickens tend to recover in 10-14 days (1, 23).

1.2. ILT Vaccination

ILT was first identified as a respiratory disease in 1925 (34) and was the first avian disease to have a vaccine created for it in 1934 by Brandly and Bushnell (5). Today there are several types of ILT vaccines including: tissue culture origin (TCO), recombinant, and chicken embryo origin (CEO) vaccines. Tissue culture vaccine virus is usually administered via eye drop, has relatively low virulence, low protective immunity, and a decreased risk for birds developing the disease. Recombinant ILT vaccines, although more expensive, provide similar coverage to that of TCO vaccines. While these vaccines have limited protective immunity, there is no risk of the birds spreading the disease and are therefore preferred over TCO vaccines. Chicken embryo origin vaccines generally provide the birds with better protection; however, these vaccines can sometimes be too virulent and can create disease in otherwise healthy flocks. The CEO vaccine can be administered through eye drop and mass (spay or water) vaccination methods. These vaccine strains are also difficult to distinguish from the naturally occurring ILTV infections due their pathogenic nature.

According to a study by Robertson and Egerton (42), chickens vaccinated against ILT via drinking water often do not develop sufficient protective immunity.

Successful drinking-water vaccination is dependent upon the virus coming into contact with susceptible nasal epithelial cells via aspiration of the virus through the external nares (42). The incorrect administration of ILT vaccines by spray may result in adverse reactions associated with deeper penetration of the respiratory tract, or excessive dosing (41, 4). Individualized vaccine application by eye-drop route was shown to provide more uniform protection but is an inefficient method of administration for large production facilities (45). Attenuated live vaccine virus which is insufficiently attenuated may also result in disease in unvaccinated chickens and is therefore a threat to biosecurity.

In addition, Guy et al (20, 19, 18) has provided evidence indicating the involvement of modified-live ILT vaccine viruses in field outbreaks. Their research suggests that as modified-live ILT vaccine virus spreads in vivo (bird-to-bird) it increases in virulence. In their studies comparing six modified-live ILT vaccine viruses and field ILTV isolates, the vaccine viruses were shown to be indistinguishable from the field isolates based on DNA restriction endonuclease analysis (20). However, during field tests, the virulence of all of the vaccine viruses was low when compared with field isolates (19). The industry needs to create a vaccine that provides the protection of a CEO vaccine, but has the low risk associated with a TCO vaccine.

1.3. Infectious Laryngotracheitis Virus (ILTV) Overview and Structure

ILTV is classified as a member of the genus Iltovirus within the family Herpesviridae, subfamily Alphaherpesvirinae (45). Infectious laryngotracheitis virus along with the psittacid herpesvirus 1 (PsHV-1), and pseudorabies virus all have a distinctive inversion in the unique long region (49). However, only the ILTV and PsHV-1 have five (A-E) unique open reading frames (ORFs) in the unique long region and are

therefore the only members of the Iltovirus genus. The virus is taxonomically identified as Gallid herpesvirus 1 (6,43).

The complete virus particle has a diameter of 195-250 nm and consists of an icosahedral (T=16) capsid shell of 150 hexons and 12 pentons surrounded by an irregular, host-derived and glycoprotein infused irregular envelope (37). Nucleocapsids may be observed within enveloped particles when penetrated by a Giemsa or hematoxylin and eosin stain. The envelope contains viral glycoprotein spikes as projections on its surface for cellular recognition.

Infection begins with the binding of virus to a cell's surface and the virus fusing with the cell membrane to enter the cytoplasm of the host cell (37). The nucleocapsid is then carried to the nuclear membrane where it fuses with the nuclear membrane. The DNA genome then enters the nucleus of the host cell and begins using the host's transcription and translation proteins to produce the alpha, beta, and gamma proteins which will then replicate the viral DNA (37). Procapsids are initially produced in the nucleus and filled with viral DNA. The procapsid then buds through the nuclear membrane. Viral glycoproteins are synthesized in the endoplasmic reticulum, and after modification by the Golgi Apparatus they enter the exocytotic pathway and are expelled into the extracellular matrix (37; Figure 1).

The ILTV DNA genome has been reported as having a guanine plus cytosine ratio of 45% (39), a value lower than many other animal herpesviruses. The DNA genome consists of a linear 148-kb double stranded molecule composed of a unique long (UL) and a unique short (US) region which is flanked on either side by inverted repeats (25, 32). In 2006 the complete nucleotide sequence of the ILTV genome was assembled from 14 different published sequences (Table 1). The assembled ILTV genome showed that the unique long (UL) region was approximately 113kb in length and that the unique

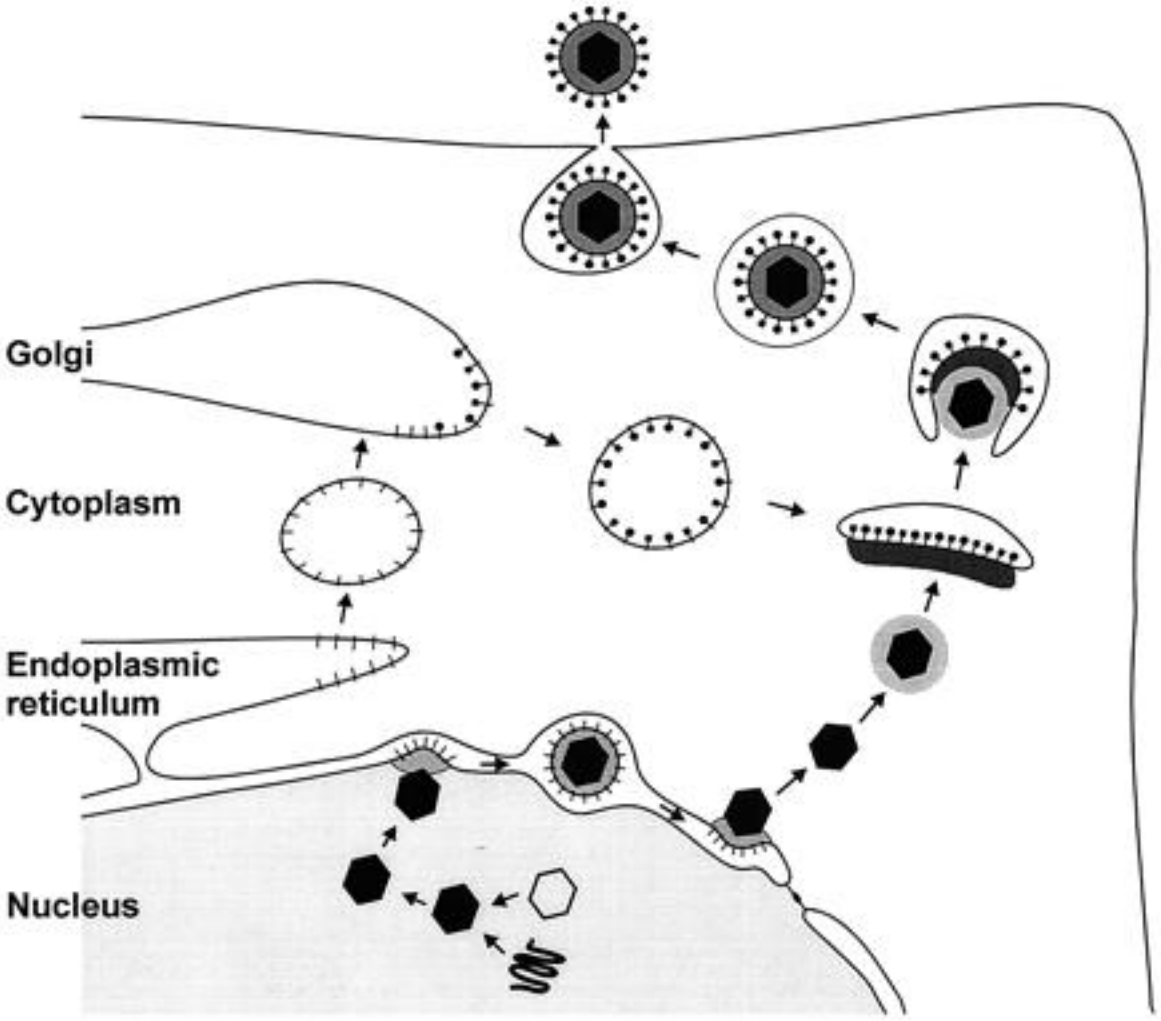

Figure 1: Mettenleiter's proposed summary diagram of herpesvirus (ILTV) egress. (37)

Table 1: ITLV Sequences used to compile the ILTV Reference Sequence.

(Accession number: NC_006623). The ILTV viral sequences used to compile the complete ILTV genome reported in Thureen and Keeler (49).

GenBank accession #	Reference	Size (bp)
U80762	Johnson et al. (26)	13700
AJ249803	Johnson et al (27)	4465
Y14300	Ziemann et al. (51, 52)	15276
D00565	Griffin and Boursnell (17)	5395
X56093	Poulsen et al. (40)	3065
AY033142	Kehu et al (unpublished data)	1360
AF168792	Johnson (unpublished data)	31332
AY033143	Kehu et al (unpublished data)	378
U06635	Kingsley and Keeler (30)	1807
Y14301	Ziemann et al (51, 52)	1854
AJ131832	Fuchs and Mettenleiter (12)	24140
X97256	Fuchs and Mettenleiter (11)	10265
L32139	Johnson et al. (26)	8364
U28832	Wild et al. (50)	18900
L32139	Johnson et al. (26)	8364
	Total	148665

short (US) region was approximately 13 kb with the two inverted repeats on either side equaling 11 kb each (49). The ILTV genome contains a total of 77 predicted open reading frames with 62 of these located in the UL region, 9 in the US region, and 3 in the inverted repeats.

Recent studies have shown that laryngotracheitis virus mutants containing deletions in genes coding for gJ, gM, and gN are still viable and that these glycoproteins are not essential for virus replication (8, 13, 14). Another study which examined ILT viruses with a double gI/gE gene deletion demonstrated that these two viral glycoproteins appear to be essential for virus replication (7). Further studies are required to identify other critical gene mutations and their affect on viral replication and pathogenicity.

1.4. DNA Sequencing

Nucleotide sequencing began in the early 1970's with the complete RNA genome sequencing of Bacteriophage MS2 by Walter Fiers at the University of Ghent (38, 10). However, these early methods such as "wandering-analysis" were tedious and unreliable. In 1967 Allan Maxam and Walter Gilbert developed a complex radioactive method of DNA sequencing based on chemical modifications to DNA and cleavage at specific bases (35, 16). This method, due to its complexity and extensive use of hazardous chemicals, was quickly replaced. In 1975, Frederick Sanger developed a simpler and more reliable method for sequencing known as "chain-termination".

The Sanger method (chain-termination) uses dideoxynucloetide triphosphates (ddNTPs) as DNA chain terminators, a single-stranded DNA template, a DNA primer, DNApolymerase and fluorescently labeled nucleotides (46). Originally the sequencing sample was divided into four separate sequencing reactions containing all four deoxynucleotides but only one of the dideoxynucleotides. PCR was then used to amplify

the DNA samples after which the samples were electrophoresed on a polyacrylamide gel with each sample in a different lane. The DNA was separated on the basis of mass and the order of bands represented the order of the bases in the sequence. Later, dye-terminator sequencing was developed, which utilized labeling of the chain terminator ddNTPs rather than the primers so that the sample could be sequenced in one reaction instead of four (47).

The Sanger method is both safe and reliable, and has been the trusted method for DNA sequencing for the past 35 years. The original ILTV reference sequence was assembled using 14 previously published ILTV sequences all of which were sequenced using the Sanger technique (Table 1). However, with more advanced technologies becoming available, high through-put sequencing is beginning to dominate the field of research.

High through-put sequencing technology allows for the sequencing of millions of strains in the same amount of time it once took to sequence a single DNA strain (16). Illumina and Helicos both use reversible terminator methods which temporarily remove the blocking group to allow polymerization of another nucleotide. Pyrosequencing (454) uses DNA polymerization, adding one nucleotide at a time and detecting the number of nucleotides added to a given location through the light emitted by the release of attached pyrophosphates (3, 33).

1.5. Applying Illumina Technology to Viral Sequencing

High-throughput sequencing technology is allowing researchers to rapidly sequence entire genomes at moderate cost, but at the expense of individual sequence lengths. For example, the Illumina platform is capable of sequencing an entire genome in one experiment; however, the genome would be sequenced in pieces each ranging from

20-50 nucleotides in length. These pieces would then need to be aligned by matching the overlapping nucleotides which, due to the amount of data being processed, requires advanced alignment software. The Illumina Genome Analyzer was originally designed for sequencing and analyzing the DNA of humans to look for mutations, but its application in other fields is quickly gaining popularity. Recently, de novo sequencing of a bacterial genome (*Staphylococcus aureus*) was conducted using the Illumina genome Analyzer to generate a paired-end read and a SSAKE software program designed to align short, overlapping, contigs (22). Using this technology to rapidly sequence, analyze, and compare viral genomes is however a novel project.

1.6. Objectives

Our goal is to be able to rapidly sequence any strain or isolate of ILTV. The objective of this research is to determine and compare the sequences of the USDA challenge virus, a chicken embryo origin vaccine virus, and Delmarva field isolates from 1985, 1995, and 2005. Analysis of these five strains will allow us to identify key differences between field isolates from different decades, as well as between field isolates and vaccine strains. Our lab will also sequence a 2005 isolate from Georgia which will act as both a geographical comparison and the lab ILTV reference sequence.

My thesis research has focused on the development of a lab protocol for high through-put sequencing and the use of this new technology to sequence both the 1985 Delmarva (632) and 2005 Georgia (1874C5) field isolates. By sequencing a 2005 (1874C5) isolate a new, more reliable, lab ILTV reference sequence was also created. The 1985 (632) field isolate was also sequenced and aligned to both the reference ILTV genome and the 1874C5 sequence for further comparison.

Chapter 2

MATERIALS AND METHODS

2.1. Chicken Embryo Liver (CEL) Tissue Culture

ILTV is propagated on chicken embryo liver (or chicken kidney) cells. Livers are collected from 14 day old chicks and placed into a 150x15mm petri dish containing 15mls of DMEM with Pen/Strep. The livers are finely minced and rinsed three times in sterile cold 1xPBS. The minced liver tissue and 25mL of 3x trypsin (warmed to 42°C) were then placed in a trypsinizing flask and gently mixed for ten minutes. After mixing, the supernatant was poured through cheesecloth and into a sterile beaker containing 10mL of chicken serum to halt the reaction. This process was repeated twice and the final suspension (~75 mL) was centrifuged at 1100 rpm for 15 minutes at 4°C and the pellet resuspended in a mixture of DMEM supplemented with 10% fetal calf serum. Using the ratio of 1mL cells: 100-110mL media, the cells were incubated overnight at 37°C and 5% CO2. The next day, the cells are infected with virus and allowed to grow for another night. The infected cells are then harvested on the third.

2.2. Viral DNA Isolation

ILTV-infected cell pellets were sonicated using the "horn sonicator" at 30% amplitude for approximately 10 seconds and resuspended in 9 volumes of lysis solution. NaCl was then added for a final concentration of 0.2M and centrifuged at 1400xG for 10 minutes. After mixing, 500uL of NaCl and 7% PEG8000 was added for every 11.5mL of lysate. The mixture was then divided into 6 nalgene ultratubes and the "surespin 630" in

room 307 WOR was used to centrifuge the sample for 30 minutes at 9,500rpm and 4°C. After drying the sample in a SpeedVac Desiccator (ThermoSavant; Fermingdale, NY), the pellets were resuspended (3:1) in 1mL TE and stored at 4°C.

After adding 20uL of 10% SDS (0.2% final concentration) to the reaction mixture, 10uL of proteinase K was added, and the solution was incubated at 37°C for 2.5 hours. The DNA was extracted 5 times with equal volumes of PIC (phenol/chloroform/isoamyl alcohol) and precipitated with 1/10 volume of 3M NaAc and 2x volume of 100% EtOH. The sample was then frozen at -80°C for 1 hour and centrifuged for 20-30 minutes at 14000rpm to remove the supernatant. The pellet was then washed with 70% EtOH (1000uL), dried, resuspended, and pooled in 400-600uL of deionized water. The DNA sample was then loaded onto the top of sucrose gradient and centrifuged in a S20/30 rotor at 18630 rpm for 18 hours at 4°C.

Using a bracket and pole stand, the bottom of each tube was punctured with an 18 gauge needle and 1mL (~14-15 drops) was added to each of the 35 labeled 2mL tubes. 20uL of each fraction was electrophoresed on an 0.8% agarose gel at 10 milliAmps overnight. The fractions containing DNA were pooled together, an equal volume of TE and twice the volume of EtOH (100%) was added to the sample and centrifuged at 25,000 rpm for 1 hour. The sample was then resuspended in 400 uL of TE, 40 uL of 3M sodium acetate and 880 uL of 100% EtOH and frozen at -20°C overnight. The next day, the sample was centrifuged, the supernatant discarded, and the pellet was washed with 70% EtOH, dried, resuspended in 100 uL TE and an EcoR1 digest was run to ensure ILTV DNA was collected.

2.3. Illumina Library Creation

The protocol accompanying the Illumina Library Creation Kit ("Preparing Samples for Sequencing Genomic DNA"; Illumina, Part # 1003806) was followed in the creation a genomic library for sequencing on the Illumina genome analyzer. The first step for preparing genomic ILTV DNA libraries for the Illumina Cluster Station and Genome Analyzer was to fractionate the DNA into 800 bp (or smaller) fragments via nebulization. Following this, the overhangs were converted into blunt end fragments with 5'-phosphorylated ends. This was achieved by the use of T4 DNA polymerase and E.coli DNA polymerase I Klenow fragment enzymes to remove the 3' overhang via exonuclease activity. These enzymes also filled in the 5' overhangs with their polymerase activity. By adding an "A" base to the 3' end of the blunt phosphorylated DNA fragments, the DNA was prepared for ligation to adapters which have a single "T" base overhang at their 3' end. To prepare the sample for flow cell hybridization, the adapters were ligated to the ends of the DNA fragments using DNA ligase and the Adapter oligo mix (provided with the Illumina "Genomic DNA Sample Prep Kit"; Catalog# FC-102-1001). To purify the ligation product and remove un-ligated adapters from the sample, a 2% agarose gel with 50mL of 1X TAE buffer was run and the region of gel that contains the desired DNA was isolated and a polymerase chain reaction (PCR) was run on the library to amplify the cDNA. This technique used PCR primer 1.1 and 2.1 (provided with the Qiagen PCR Amplification Kit- Qiagen; Valencia,CA), and was carried out in a thermal cycler. Following initiation at 95°C for 2 minutes, ten cycles of denaturation (65°C for 30 seconds), elongation (Tm-5°C for 30 seconds), and elongation (72°C for 1 minute) were performed.

2.4. Illumina Genome Sequencing

After PCR amplification, the library was validated and clustered using the Illumina Genome Analyzer. To validate the library, another 2% agarose gel was prepared using 10% of the library to ensure that the size range of DNA was as expected from the ILTV DNA isolation step described previously. Another validation technique used was to measure absorbance at 260nm (should be between 500-1000ng of DNA) and to measure the 260/280 ratio (approximately 1.8) using a spectrophotometer (Nanodrop Technologies; Wilmington, DE).

To cluster the library, the "Cluster Station" took the DNA library and created "clonal clusters" for the Genome Analyzer by amplifying single-molecule DNA fragments. These fragments were bound to the flow cell surface via base pairing between the 3' A bases added during library creation and the T base adapters. The Genome Analyzer then ran single-base extension cycles, taking pictures after each cycle to see in which order the bases were added (Figure 2). These images are commonly known as "starfields", due to their resemblance of a night sky (Figure 3). The software then analyzed the size and shape of each added nucleotide, and began to compile a sequence order for each cluster. Once the Genome Analyzer finished the first sequencing read, the paired-end module then directed the re-synthesis of the original templates and began the second round of cluster generation. The system software of the Genome Analyzer then converted the raw image data into readable sequence and compiled the cluster sequence data into contigs.

2.5. ILTV Sequence Analysis

The contigs generated from the Genome Analyzer were aligned to the ILTV reference sequence in either Genome Studio (Illumina) or the CLC Genomics Workbench (CLCbio; Cambridge, MA). These software packages determine the amount of reference

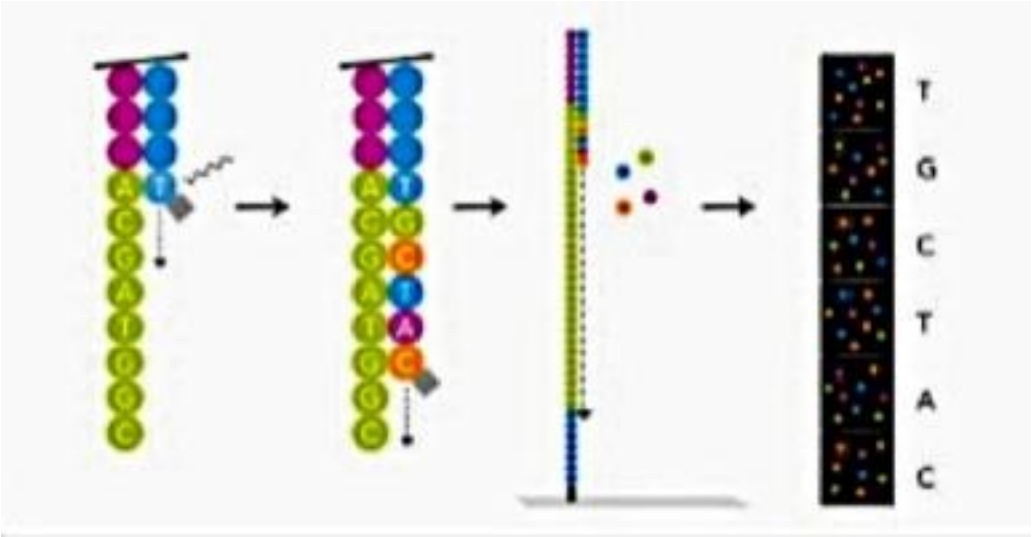

Figure 2: Nucleotide Addition -The addition of A bases allows for the attachment of the DNA fragment to the flow cell which contains a T "primer". The Illumina sequencer uses a reversible terminator which temporarily removes the blocking group to allow the polymerization of an additional nucleotide. Each cycle adds one nucleotide at a time and the sequencer takes a picture after each cycle to show which base was added. The result is the "star field" image shown in Figure 3.

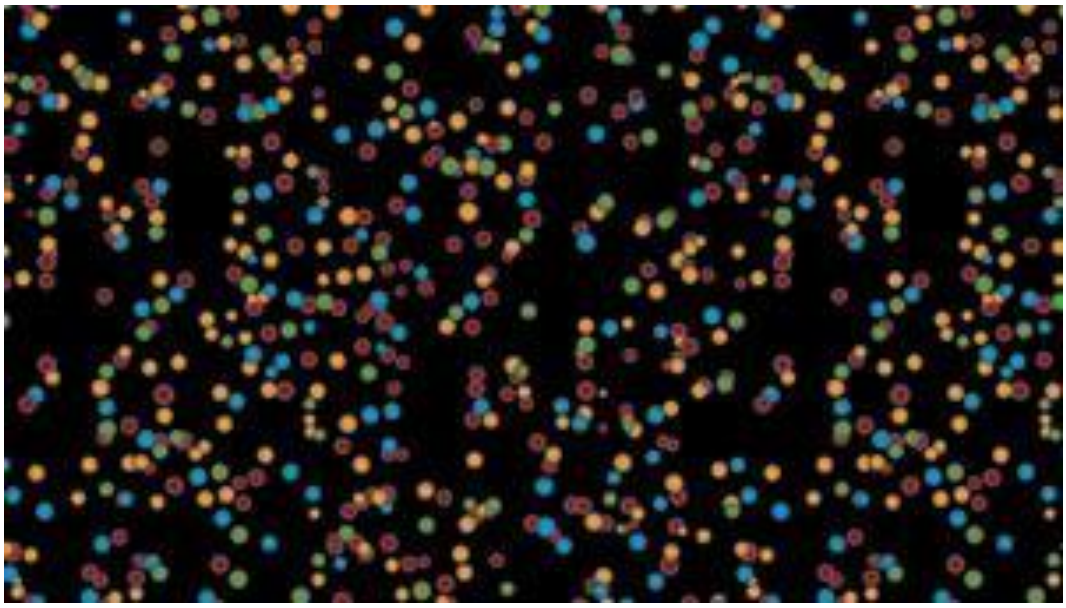

Figure 3: Starfield. An example of the "Starfield" generated by the Illumina Genome Analyzer after the addition of each new base.

sequence that was covered, single nucleotide polymorphisms (SNPs) between the reference and Illumina sequence and any regions of zero coverage. Regions of reference sequence that were not aligned (none of the contigs created matched that region) are considered "gaps in sequence" and were further investigated.

Genome Studio requires that the sequence and imaging data be organized into the Consensus Assessment of Sequence and Variation (CASAVA) format. The CASAVA conversion software program converts raw image data into base calls for further analysis. Uploading these CASAVA files onto Genome Studio allows the user to view the viral genome and identify polymorphisms between the sequenced strain and the reference genome (Figure 4). However, this program does not provide an efficient method for identifying regions of zero or low sequence coverage. Therefore, to identify these regions, individual contigs were copied into Excel. Using Excel the contig lengths and positions were subtracted from each other to determine where they no longer overlapped (gaps).

Genome Studio also has no method for using the Illumina single-read sequence data to create a de novo sequence. In order to create a new sequence using Illumina data, a PERL script was developed (Timothy Keeler, 2009) which replaces the nucleotides in the published reference sequence (NC_006623)that Genome Studio identified as SNP positions. This protocol creates a text document containing the reference sequence with all identified SNPs replaced but will not remove or identify gaps in Illumina's coverage of the reference genome.

The CLC program was also used to identify gaps and SNPs in the ILTV sequence. The Genomics Workbench allows users to align both raw Illumina sequence data and imported NCBI sequences to generate an aligned Illumina sequence using the "reference assembly" option (Figure 5). This software uses a smaller "sequence.txt"

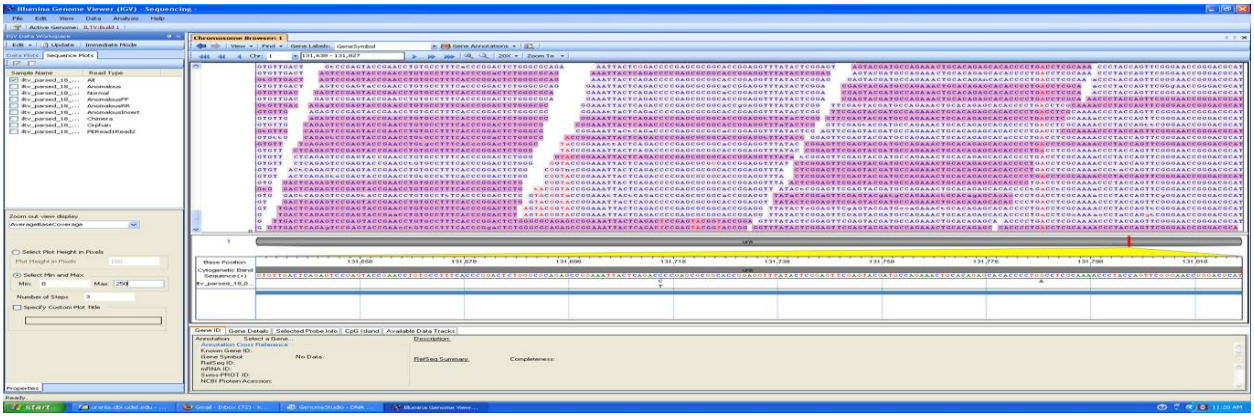

Figure 4: Genome Studio Screen Shot- A screen shot of Genome Studio zoomed in to the nucleotide level. Those nucleotides which are highlighted in red represent SNPs. The reference sequence is across the x-axis with any SNPs listed below the reference. The depth of coverage is larger than represented, scrolling up will allow users to view all nucleotides sequenced at a given location.

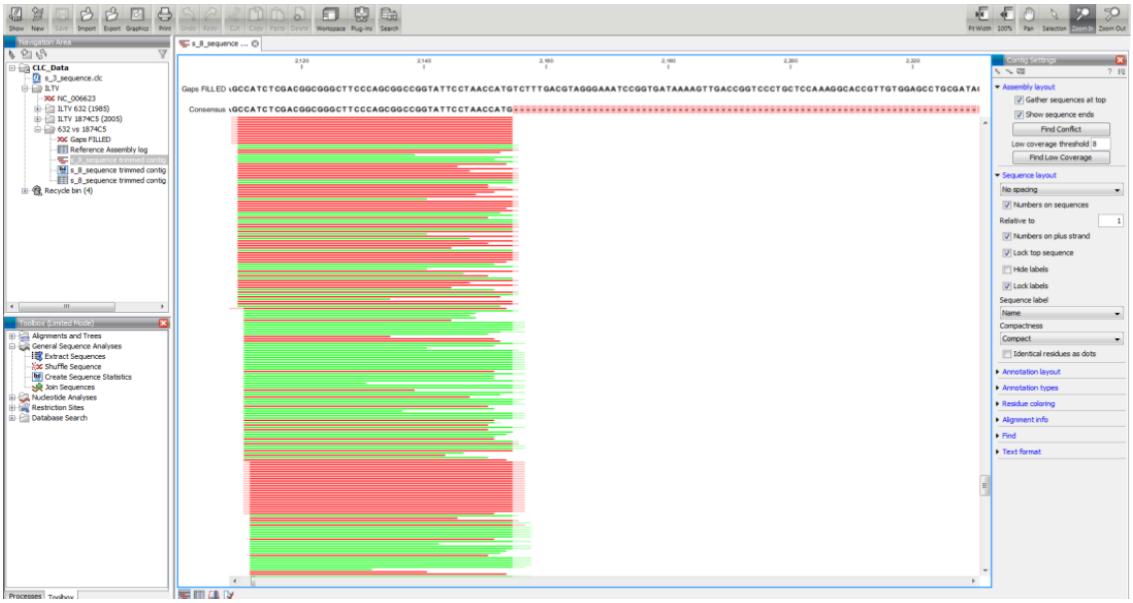

Figure 5: CLC Genomic Workbench, 1+ Zoom Screen Shot. - A screen shot of the CLC Genomics Workbench zoomed into 1+ the nucleotide level. At this zoom, users are able to see the nucleotides of both the reference (above) and the consensus sequence (below). The SNPs will be listed in red with any missing nucleotides (deletions/gaps) listed as red dashes as seen below. As with Figure 4, the depth of coverage is deeper than seen and can be viewed by scrolling down.

Illumina file, and does not require CASAVA formatting. The alignment report identifies the percentage of a sequence that aligns to the given reference, the number of nucleotides which aligned to the reference, as well as the location and length of regions with zero coverage (either gaps in read data or deletions). To identify SNPs between the reference and Illumina Sequence, the "SNP detection" program is run. This program creates a table indicating where the SNPs are, what the mutation is, and the percentage of times Illumina sequenced a SNP for that given location.

2.6. Illumina Gap Correction with PCR

"Gaps", or regions of DNA not sequenced by Illumina but present in the reference sequence, are generally regions with a complicated 3 dimensional DNA structure, a large mutation that Illumina cannot identify as part of the reference sequence, or a legitimate deletion in the viral genome sequence.

As described earlier, there are two methods for identifying these gaps in sequence. The first (using Genome Studio) was to use an Excel spread sheet and identify non-overlapping contigs. The second was to use the CLC genomic workbench. Regardless of which method was used, the validity of the sequence in these areas was checked with PCR amplification and Sanger sequencing.

To identify primers for PCR, 100-200 bases from either side of the gap (Figure 6) were selected from the Illumina sequence and copied into the "Primer3" program (Primer3, Howard Hughes Medical Institute and NIH-NHGRI) with "X"s substituted for the gap itself. Primer3 identified both a forward and reverse primer for sequencing the proposed region of DNA. Following the selection of appropriate primers, PCR amplification is performed to increase the amount of DNA being sequenced.

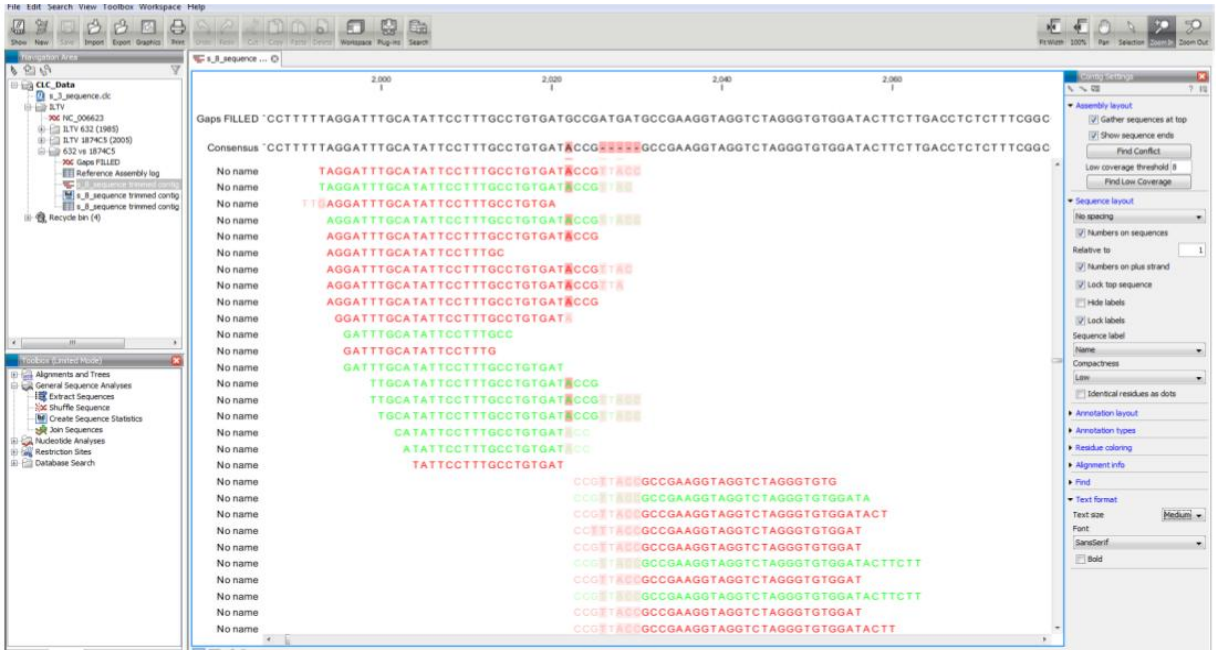

Figure 6: CLC Primer Identification CLC will label the "zero coverage regions" (gaps) as dashes highlighted in red. To find these, there is a "search for low coverage region" tab to the right. Copy and paste the Illumina sequence into primer3 substituting the dashes with "X" when attempting to identify primers for PCR sequencing.

ILTV DNA was amplified in a reaction containing 0.5ul of Forward Primer, 0.5ul of Reverse Primer, 45ul of Platinum PCR Supermix (Invitrogen), 3ul of water, and 1ul of ILTV DNA. The polymerase chain reaction was run in a thermal cycler (Applied Biosystems) and began with the initialization phase at 95°C for 2 minutes. Then, a 40 step PCR cycle was performed at 95°C for 30 seconds (denaturation), an annealing temperature at the melting temperature of the primers minus 5°C (approximately 55°C) for 30 seconds, and an elongation temperature of 72°C for 1 minute, followed by a final elongation step at 72°C for 10 minutes.

Chapter 3

RESULTS

3.1. Creating Viral DNA

Following the procedure outlined in the Material and Methods section for the 632 (1985 DE isolate) strain, ILTV DNA was purified from isolate "632". Purified 1874C5 (2005 GA isolate) ILTV DNA was provided by Maricarmen Garcia, Ph.D. and the University of Georgia.

3.2. Illumina Library Creation

After preparing the 2005 isolate (1874C5) using the "Preparing Samples for Sequencing Genomic DNA" protocol (Illumina; Part # 1003806), a 175bp purified ligation product was isolated and amplified using PCR. This adapter-modified DNA was then amplified using PCR and the library was validated via gel electrophoresis and spectrophotometer analysis. Spectrophotometer analysis indicated a 260/280 ratio of 1.89, and 19.6ng/uL. The gel indicated a product approximately the same size as was excised (Figure 7).

Following the first 632 single-read ligation for a single-read Illumina preparation, a band of DNA was isolated in the 175-250bp range (under the dye front), and amplified using PCR. This adapter-modified DNA was not validated with gel electrophoresis due to a dye front covering what should have been the 175-250bp range. Also, spectrophotometer analysis indicated the 260/280 ratio was 1.5 and the sample had a concentration of 17.8ng/uL. Because these results were so poor, another section of

Figure 7: Gel picture of library validation for 1874C5.

Lane: M = marker, Lane 1= 1874C5 Library

DNA was excised above the dye front at 500bp and amplified using PCR. This adapter-modified DNA was then successfully validated by gel electrophoresis and spectrophotometer analysis with a 260/280 ratio of 1.99, and 27.4ng/uL (Figure 8).

3.3. Sequencing Results

The sequencing of the 1874C5 isolate yielded 6,122,687 nucleotides of ILTV which aligned to the reference sequence. The mean read length was 41.5 bases, and the mean depth of coverage (number of times each position was sequenced) for the 1874C5 isolate was 41.07 nucleotides across the entire genome.

432,786,937 of the 632 ILTV isolate's sequenced nucleotides aligned to the reference sequence. The mean read length for this sequencing attempt was 44.24, and a mean depth of coverage of 2,869.02 nucleotides across the entire genome was reported.

When aligning the 632 ILTV nucleotides to the corrected 1874C5 sequence, a total of 437,147,551 nucleotides were positioned. The mean depth of coverage after aligning to this sequence was 2,903.96 nucleotides. The depth of coverage at each position for 632 aligned with corrected 1874C5 sequence is shown in Figure 9.

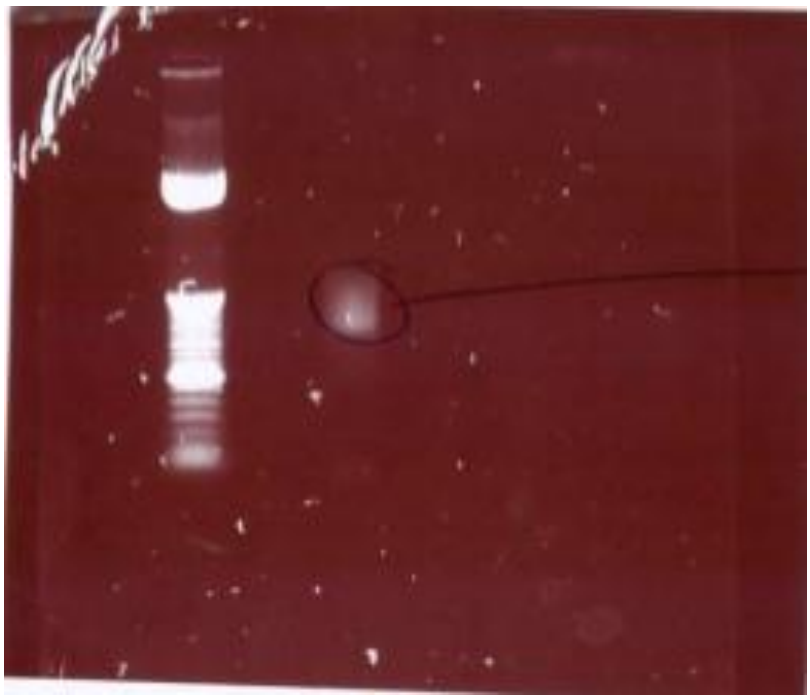

Figure 8: Gel picture of library validation for 632.

Lane M= Marker, Lane 1= 632 Library

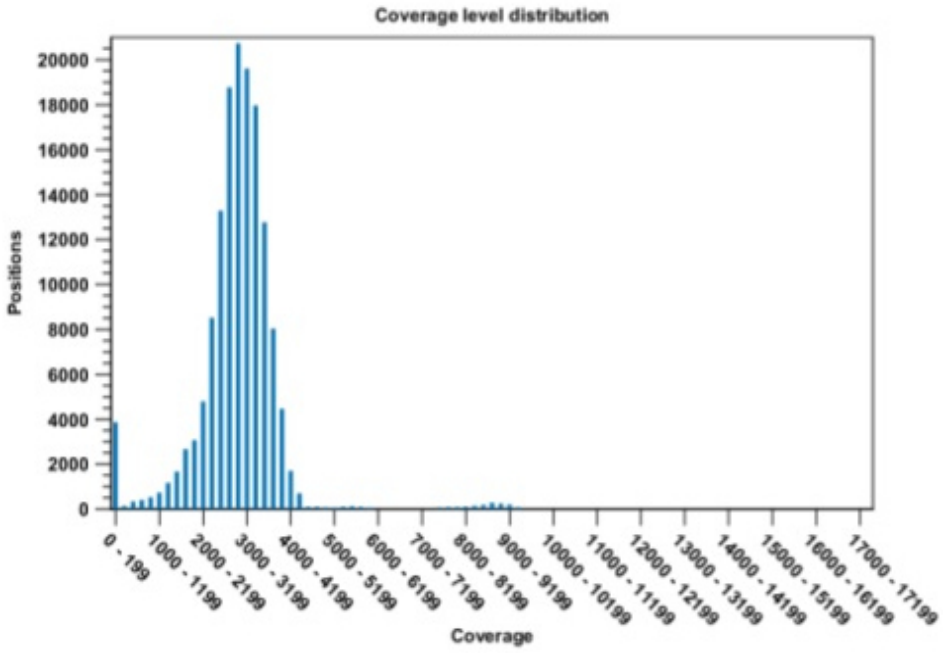

Figure 9: Coverage level distribution of 632: This figure shows the distribution of coverage for the 1985 genome after using the Corrected 2005 sequence as a reference. The number of times a certain nucleotide was sequenced (coverage) is shown across the x-axis, while the number of positions that have that amount of coverage are distributed across the y-axis.

3.4. Genome Studio and CLC Workbench Analysis Results

Comparison of the 2005 Georgia strain (1874C5) and reference strain with Genome Studio revealed that the sequences were 98.9% identical. A total of 529 SNPs were identified with 22 in the unique short region. There were also 18 nucleotides added and 17 nucleotides deleted in the 2005 isolate sequence. The CLC program only identified 294 SNPs between the reference and 2005 strains which increased the homology of the two strains to 99.2%. The same 22 SNPs were identified in the unique short region with a higher certainty (Table 2).

Using CLC to compare the 1985 Delaware strain (632) with the NCBI reference sequence revealed a 97.3% identity. A total of 231 SNPs were identified with only 2 of them located in the unique short region. A total of 3,836 nucleotides were also missing from the 632 sequence (gaps/deletions). 3,333 of these missing nucleotides were associated with one gap from base pairs 2155 through 5487 (Table 2).

CLC comparison of the 1985 Delaware strain (632) and the corrected 2005 Georgia strain (1874C5) revealed the two to be 97.6% identical. CLC also identified 128 total SNPs, 24 of which are located in the unique short region (Table 2). A total of 3,481 nucleotides were also missing from this sequence alignment with the same 3,333 bp gap identified.

3.5. Identifying and Correcting Gaps in the 1874C5 Sequence

Analysis of the Illumina sequencing with the Genome Studio software identified 529 SNPs throughout the entire ILTV genome. Further analysis with the aid of Excel revealed 11 regions, a total of 361 nucleotides, where Illumina could not match nucleotides to the reference sequence (Table 3). Illumina identified these regions as gaps which were then investigated further with PCR and Sanger sequencing analysis. Following PCR, each of the amplified portions of DNA were isolated on a gel and gel

Table 2: Comparison of the NCBI reference, 2005 Isolate, and 1985 Isolate using the CLC Genomics Workbench.

Strain	Aligned against	Consensus Length	Nucleotides Aligned to Sequence	Homology	US SNPs	Total SNPs
1874C5	NC_006623	147773	6122687	99.2%	22	294
632	NC_006623	144851	432786937	97.3%	2	231
632	1874C5	144951	437147551	97.6%	24	128

Table 3: The results of the PCR gap sequencing for the 1874C5 (2005 Georgia) isolate.

Each gap's position, primer, and size is listed along with the results of the sequencing analysis.

Gap	Position	Primer	Predicted Gap	Actual Ins/Del	Identified SNPs
11	690-701	TTCCTGTGGGTCTCTTCCAG	11	0	11
		AATTCAGCCGAGGATTTGG			
95	3373-3468	CTACGTGACTTGCCAGCATC	95	2	116
		CAATATCACAGGCATTAGTCAG			
12	4663-4675	CCTTAGGCGACTTCCACTGA	12	11	12
		GCGAGTGCATGATCGAACTA			
4	7959-7963	TGCCTCTTCGGACTTGGATA	4	0	3
		GACAAAAGATCGCCCTGGAT			
11A	10612-10623	GACGGTTTTTGCGGGTATTA	11	0	2
		TCCCCGACTCTCGAAAATTA			
32	10695-10727	CACCACGTTCATCAACCAAG	32	0	4
		TTGCCATCTCTGTGACTGGT			
29	10911-10940	ACGTAGGATGGCACCAATTC	29	0	4
		GCCAGAACATTGTGGGACAT			
1	12108-12109	AACGAGGCTTCCCCATTC	1	0	1
		CACGCCGAGGTCAGAATC			
5	12198-12213	See gap 43	15	0	0
		See gap 43			
43	12269-12312	ATGAGATCTGCGGCGAATAC	43	0	2
		CGTTGTGTATGCGGTTTCAT			
108	13383-13491	CCCGTAATATCAGCCTCGAA	108	0	0
		ATCCCACTGGCCAAAGAAC			

purified (example Figure 10A and 10B) and the spectrophotometer was used to obtain the 260/280 ratio and the concentration of DNA per sample. These results were used to ensure successful Sanger sequencing which was conducted by Bruce Kingham at the Delaware Biotechnology Institute (DBI-Newark, DE).

The results of Sanger Sequencing were then used to correct for errors in Illumina's sequencing across these regions. According the PCR sequencing, Illumina was correct in sequencing a deletion from the reference sequence only once. While the remainder of the proposed gaps did have nucleotides in the given location, many of the regions sequenced had both low coverage and a high concentration of SNPs (Table 3).

3.6. Identifying a 3,333bp deletion in the 632 (1985 DE isolate)

The analysis of the 632 (1985 DE Isolate) ILTV strain was done on the CLC Genomics Workbench program and yielded the identification of 231 SNPs when compared with the reference sequence, but only 128 SNPs when compared to the 2005 Georgia isolate.

When aligned to the corrected 2005 sequence, Illumina identified a total of 18 gaps or 3,481 missing nucleotides. 3,333 of these missing nucleotides (1 gap/deletion) are located in the region from base pairs 2155 through 5487. These missing nucleotides account for 95.8% of the missing sequence between the 2005 and 1985 sequence. Following primer identification, PCR, and gel purification, a section of DNA approximately 530 bp long was isolated and sequenced using Sanger sequencing. The results of both gel purification and Sanger sequencing indicated that the 3,333 bp gap was in fact a deletion in the 632 genome, and that the Illumina sequencing was correct in not sequencing the nucleotides. Illumina also identified a smaller gap (5 bases) only 124

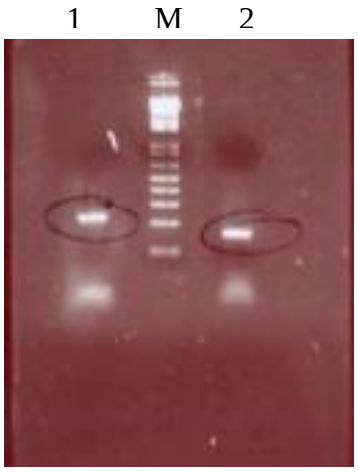

Figure 10A: Gel picture of gap 4 and 12 PCR amplification.

Lane 1 = Gap 4 PCR product Lane M = Marker Lane 2 = Gap 12 PCR product

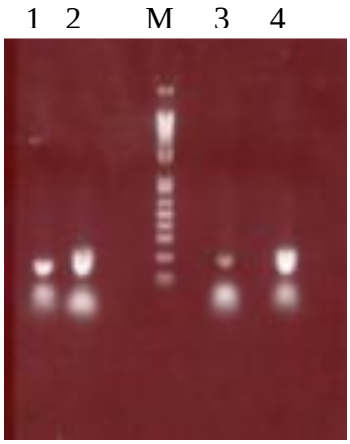

Figure 10B: Gel picture of gaps 1, 29, 32, 108 PCR amplification.

Lane 1 = Gap 1 PCR product, Lane 2= Gap 29 PCR product, Lane M= marker,
Lane 3= Gap 32 PCR product, Lane 4= Gap 108 PCR product

nucleotides before the larger 3,333 bp gap. By sequencing across the 3,333 bp gap, the 5 bp gap was also sequenced. In this instance, five nucleotides were present at the location of the "gap" identified by Illumina, however, all 5 nucleotides were SNPs (Table 4).

Table 4: Results for PCR gap sequencing for the 632 (1985 DE) isolate.

Each gap's position, primer, size of gap, and the results of the sequencing analysis. Primers for the 3,333 bp gap were used to fill both the 3,333 bp and 5 bp gaps in the 632 sequence. The 3,333 bp gap was identified as a true deletion in the sequence, while the 5 bp gap was identified as 5 SNPs which Illumina did not sequence. One additional SNP was found between the PCR and Illumina sequences.

Gap	Position	Primer	Predicted Gap	Actual Ins/Del	Additional SNPs
3333	2155-5487	TAGGGTCATTGTCGGAGCTT	3333	3333	1
		ATCCGCAGTGTTACCTGGAC			
5	2026-2030	See gap 3333	5	0	0
		See gap 3333			

Chapter 4

Chapter 4

DISCUSSION

4.1. Use of Illumina Technology for Viral Sequencing

Illumina High-Throughput Genome Sequencing is a reliable and cost-efficient method for large sequencing projects. While previous efforts to sequence complete genomes took many months and the efforts of multiple labs, whole-genome sequencing can now be conducted in weeks via high through-put sequencing technology.

Although this sequencing technology is not completely error proof, with the inherent bias associated with aligning any sequence to a reference (non-de novo sequencing), the quantity and speed at which the data is generated is a great asset for researchers in many fields. For projects aiming to compare various strains of sequence, being able to rapidly sequence a genome thousands of times over is now necessary to ensure the validity of a sequence. PCR sequencing should be used to verify regions of questionable validity, such as regions with no coverage, low coverage, or where problems have been known to occur in the past. Because these troublesome areas are often regions with low coverage, PCR is also likely to identify more SNPs.

Due to the low coverage associated with the Illumina sequence in the surrounding area of gaps, any high quality (double stranded) PCR sequence was

considered more trustworthy than poor quality Illumina sequence. Therefore the final "corrected Illumina Sequence" is a combination of both the Illumina and PCR sequences.

4.2. Sequencing Advancements

In only four decades, DNA sequencing technology has gone from the sequencing of a few hundred nucleotides to hundreds of millions (possibly billions) of nucleotides. Technological advancements have made whole-genome sequencing a reality, and allowed researchers to compare entire genomes in search of abnormalities. Once, simply sequencing a genome was considered unbelievable. Today, for any research to be considered valid, the depth of coverage must be investigated. While sequencing technologies will undoubtedly continue to get faster and more powerful, further advancements in library creation and analysis software are required so that researchers are able to efficiently analyze data that they can trust.

4.3. Analysis Software

Illumina's sequencing technology is efficient and extremely powerful; however, their analysis software (Genome Studio) lacks the basic functions our lab requires to rapidly analyze and compare isolates. Currently the software has no function associated with de novo sequencing, which would eliminate bias when aligning a sequence, and no method for efficiently identifying the location or length of any zero coverage regions.

The CLC Genomics Workbench avoids CASAVA formatting and allows users to download sequences directly from NCBI. It also has an efficient method for identifying both SNPs and regions of low or "zero" coverage. By downloading reference sequences directly to the CLC workbench and aligning the raw Illumina sequencing data to the reference, users are able to reduce the amount of memory required to view the

sequencing data. Avoiding use of the PERL program to create an Illumina consensus sequence from a reference is also helpful so that users are not forced to learn yet another computer program. CLC also has a "report" function that provides the statistics of the Illumina alignment in which zero coverage regions are organized into a table with locations and lengths of each identified. The ability to rapidly compare multiple sequences is an invaluable tool as the lab progresses and more strains of ILTV are sequenced.

4.4. Comparison of Reference, 1985, and 2005 Field Isolates

A comparison between the published ILTV reference sequence (NC_006623), a 2005 ILTV Georgia field isolate (1874C5), and a 1985 ILTV Delaware field isolate (632) was conducted using the CLC Genomics Workbench. This comparison revealed a total of 294 SNPs between the 2005 isolate and the reference sequence, 231 SNPs between the 1985 isolate and the reference sequence, but only 128 SNPs between the corrected 2005 sequence and the 1985 sequence. With greater than 97% of the viral genome remaining constant over a 30 year time period and between states as distant from each other as Delaware and Georgia, it can be concluded that the ILTV genome is well conserved across both time and space.

However, CLC did identify a major (3kb) deletion in the Delaware field isolate from base pairs 2155 through 5487 (Table 4). This deletion does not encompass any genes currently known to be associated with ILTV. However, the biological significance of such a major deletion requires further investigation. To determine if the deletion is endemic to Delaware, further sequencing of Delaware field isolates should be conducted across variable years. Delaware isolates being sequenced should also include

vaccine strains to determine if the deletion is a common marker indicating the strain's evolution from a vaccine virus.

The next step towards completing a comparison between ILTV isolates would be to finish PCR sequencing of gaps in the 632 sequence and being sequencing additional Delaware field isolates from different eras.

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