ORF VIRUS GENOME SEQUENCING

A Thesis

by

DANIEL LUCAS HEARE

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Sara Lawhon
Committee Members,	Luc Berghman
	Michael Criscitiello
Head of Department,	Ramesh Vemulapalli

December 2019

Major Subject: Biomedical Sciences

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ABSTRACT

Orf virus (ORFV) is an ancient Parapoxvirus that causes substantial economic loss worldwide to sheep and goat producers. This virus causes a disease known as Contagious Ecthyma or more commonly “Soremouth” because it most commonly presents itself on the lips and mouth of sheep and goats. Soremouth makes it difficult for animals to eat and drink, therefore leading to weight loss or failure to gain weight resulting in production losses. The primary victims of the disease are suckling lambs and kid goats. These animals have immature immune systems increasing their risk of infection and are at risk for dehydration and weight loss as the lesions in the mouth affect their ability to nurse. ORFV is a zoonotic virus that can transfer from sheep and goat to humans and other species. ORFV has immunomodulatory capabilities as the virus encodes a synthetic interleukin-10, an immune down-regulator. It also inhibits other immune activation pathways such as the Toll-like receptors. ORFV is an ideal target for vaccination because of its immunomodulatory features and because the infection is generally self-limiting. A vaccine is currently under development by Texas Vet Lab, Inc. and the genome of the virus used for the development of this vaccine is sequenced and examined here. The goal of this study was to allow comparisons between the current vaccine candidate and other sequenced ORFV genomes.

DEDICATION

I would like to dedicate this thesis to my family. My family has been essential throughout the course of this research and degree. My wife, Hannah, supported me unwaveringly through this process. She was always looking for ways to be helpful anytime she could. My son, Archer, motivated me more than he will ever know. Something about becoming a parent throughout this journey gave me more of a reason to complete this journey than I had when I initially began. My parents, Virginia and Bruce Heare, led me through this process with motivational and emotional support. Trudy and Jeffery Bozeman, thank you for always being willing to lend a hand anytime I needed. To the rest of my family and friends: I could not have done this without you. Thank you to each and every one.

ACKNOWLEDGEMENTS

I would like to thank my employer Texas Vet Lab, Inc. General Manager James Harris, and Assistant Manager Dale Weise, for the research guidance and sponsorship in research. Thanks also goes to my colleagues at Texas Vet Lab, Inc.

Special thanks go to my committee chair Dr. Sara Lawhon, and my committee members, Dr. Michael Criscitiello, and Dr. Luc Berghman at Texas A&M University in College Station for the research guidance and support as well. Ms. Sara V. Little provided invaluable guidance, support, and assistance with the sequencing and assembling the orf genome. Thank you Dr. Andrew Hillhouse your help in sequencing of the orf virus and all technical assistance throughout that process. Thank you Dr. Kranti Konganti for the technical assistance with sequence analysis and assembly.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Drs. Sara Lawhon, and Michael Criscitiello of the Department of Biomedical Science, and Luc Berghman of the Department of Poultry Science. In addition to my committee, Dr. Andrew Hillhouse at Texas A&M University and his lab were involved in the sequencing of the orf virus genome.

Ms. Sara V. Little was an integral part of the sequencing and assembly of the orf virus genome. She graciously provided help with assembly and analysis the orf genome. Drs. Kranti Konganti and Laura Bryan provided insights for removing host gene sequences from the gene sequencing data and viral genome assembly.

Funding

Graduate study was privately funded. All research was funded by Texas Vet Lab, Inc. Support for reagents and supplies for sequencing came from Drs. Sara Lawhon and Andrew Hillhouse.

NOMENCLATURE

ANK	Ankyrin repeat domain-containing protein M-T5
APCs	Antigen-presenting cells
CBP	Chemokine binding protein
CFA	Complete Freund's adjuvant
CPD	Contagious pustular dermatitis
DNA	Deoxyribose nucleic acid
dsRNA	Double stranded ribonucleic acid
EEV	Extracellular Enveloped Virus protein
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GATU	The Genome Annotation Transfer Utility
GBP1	Type II interferons
GIF	Granulocyte-macrophage colony-stimulating factor, Interlukin-2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon-γ
IL- Iβ	Transcripts for interleukin Iβ
IL-1	Interleukin 1
IL-3	Interleukin 3
IL-10	Mammalian Interleukin 10
IKK	IκB kinase
MHC-II	Major histocompatibility complex class II

mRNA	Messenger ribonucleic acid
MXA	Type I interferons
NCBI	National Center for Biotechnology Information
NK cells	Natural killer cells
NFkB	Nuclear factor kappa B
ORFV	Orf virus
PEDV	Porcine epidemic diarrhea virus
PRR	Pattern recognition receptors
q PCR	Quantitative polymerase chain reaction
TIGSS	Texas A&M Institute for Genome Sciences and Society's
TNF-α	Tumor necrosis factor-α
TVL	Texas Vet Lab, Inc.
vIL-10	Viral Interleukin 10
ViPR	NIAID Virus Pathogen Database and Analysis Resource

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1. INTRODUCTION

Orf virus (ORFV) is a highly contagious virus in the *Parapoxvirus* genus of the family *Poxviridae* (1). This virus causes a disease known as contagious pustular dermatitis (CPD), contagious ecthyma, scabby mouth, or soremouth in sheep and goats across the globe (2). The virus is rarely fatal but suckling lambs and kid goats are more susceptible to ORFV infection than older animals and are more severely affected as the lesions in the mouth can inhibit nursing leading to dehydration and decreased food intake (3). The antibodies derived from colostrum are not adequate for protection of animals from infection (4). Additionally, young animals are learning to graze, and, in this process, they can incur minor abrasions on their lips, tongues, gums, and noses. Some forages cause abrasions on sheep and goats which provides entry for this dormant virus in the sanctuary of the ground for infection of these unsuspecting animals. Lesions occur at the location of infection typically on the lips and nose or any other area of damaged skin (5). The lesions start as a raised pustule or blister and transform into a raised scab. Infectious scabs can occur anywhere there is an abrasion on the skin such as on the ears, feet, or udders of lactating ewes (6). Lesions last anywhere from two to eight weeks (7). ORFV is a highly stable, double stranded DNA virus that is 134 to 139 kbp with 130 putative genes (8). ORFV replicates in the host cell cytoplasm and encodes for its own DNA replication machinery (7). ORFV is zoonotic and can spread from sheep and goats to humans and other species (9). Although rare, there has been at least one case of human to human transmission (10). ORFV can infect camels, Japanese serows, musk ox, reindeer, cattle, alpacas, big horn sheep, Sichuan takin, domestic Shetland

sheep, deer, pronghorn antelope, wapiti and seal squirrels, as well as red deer (11-13). ORFV can remain infectious for up to 15 years if it is dry and at room temperature but can be inactivated in 30 minutes at 60 degrees Celsius (13). These characteristics make it highly difficult, if not impossible, to eliminate infectious virus particles from the environment once they have been shed from an infected animal. It has been documented that a pasture that has not held infected animals in it for an extended period can still be a source of infection for naive animals (14).

1.1. Diagnosis of Orf Infection

Orf virus infections are typically diagnosed in sheep and goats that present with characteristic crusting, proliferative lesions of the mucocutaneous junctions of the mouth and nose particularly in young sheep and goats. Confirmation of infection typically involves molecular testing for detection of viral DNA or serologic testing for the presence of antibodies. ORFV and antibodies to the virus can be detected by several methods. The primary method is real time qPCR monitoring for presence of viral DNA sequences (15, 16). This method is considered to be 100% sensitive and specific (17). Real time qPCR can be quantitative if the test on a patient sample is performed in conjunction with a standard curve containing known amounts of virus particles present in a sample (17). Determining the concentration of virus or viral load in a patient specimen provides a quantifiable measurement of the severity of an infection. Detection of antibodies to the infection is typically done using enzyme linked immunosorbent assays (ELISA) and other methods such as western blots (18). An ELISA allows

confirmation of the infection in patients with clinical signs consistent with ORFV infection and allows detection of the infection in asymptomatic animals (19). Knowledge of recent exposure of animals to ORFV is important not only for protecting naïve animals but for preventing contamination of a premise. Serum neutralization assays are not commonly used for detection of ORFV (20).

1.2. Host Virus Interaction

The orf virus is an epitheliotropic Parapoxvirus that is transmitted from animal to animal either through direct contact or through contact with virus-contaminated materials in the environment (14). The virus is unable to penetrate healthy skin (7). For the virus to infect an animal there must be some injury or abrasion of the skin in the mouth, nose, or elsewhere. These abrasions do not have to be large. Even a small thorn prick offers access to the virus. The virus enters the host through the injured skin and replicates in epithelial cells. The host responds through recognition of virally infected cells by the innate immune system. The pattern recognition receptors (PRR) recognize virions at the plasma membrane, cytosol, or at the endosomes (21). Signaling through these receptors triggers cytokine expression, which activates the innate immune system. There are several pro-inflammatory cytokines stimulated by the signaling through the PRR including interleukin 1 (IL-1), tumor necrosis factor (TNF), and type 1 interferons. In normal cells, the type 1 interferons activate and cause the release of nuclear factor kB (NFkB) and interferons (21). NFkB is important because it is responsible for controlling transcription of DNA, cytokine production and cell survival in response to

environmental stimuli through receptors. Briefly, in uninfected cells, when a signal binds to a receptor, the enzyme IκB kinase (IKK) is activated and in turn phosphorylates the IκBα resulting in dissociation of IκBα from NFkB. This activates NFkB which is then able to move into the cell nucleus where it binds to specific DNA sequences and results in transcription of mRNA (22). Although not fully understood, the ORFV functions by inhibiting activation of IKK thereby blocking activation of NFkB resulting in decreased expression of pro-inflammatory cytokines regulated by NFkB. Without these pro-inflammatory cytokines, the adaptive immune system is not activated, and memory cells are not produced.

The next step is accumulation of natural killer cells (NK cells), neutrophils, and dendritic cells (7, 23). These three cell types are the baseline response to all virus infiltration. They cause inflammation and dendritic cell recruitment. NK cells and other cytotoxic cells come into the infected area and kill the virally infected cells (24). Dendritic cells are professional antigen presenting cells (APC). This is the pathway in which the adaptive immune system is then alerted and able to assist in the removal of the virus infection. APC's present epitopes of the virion to cytotoxic T cells and B cells. Recognition of these epitopes is crucial for the adaptive immune response. The B cells once presented with an antigen can make antibodies against these antigens and T cells secrete cytokines to recruit and start the proliferation of more immune cells (25, 26). In ovine infections, messenger RNA (mRNA) transcripts for interleukin Iβ (IL- Iβ), interleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), (TNF-α) and interferon-γ (IFN-γ) are present and CD4+ T-cells predominate in the

afferent lymph draining from the site of ORFV replication (27, 28). The CD4+ T-cells are the major source of GM-CSF and IFN-y suggesting that the ORFV initiates a Th1 helper type response (27, 28).

1.3. Immune Evasion

The ORFV can repeatedly infect the same host even though there is a substantial inflammatory immune response (29-31). Although, the same animals can be infected repeatedly, as the animals mature, immunity increases, and the severity of lesions decreases. The ORFV has several unique defense mechanisms that allow it to counteract host immune responses including several immunomodulatory proteins. These proteins include: 1) a viral homologue of ovine vascular endothelial growth factor (VEGF) (32); 2) a viral homolog of ovine interleukin 10 (vIL-10) (29); 3) an interferon resistance gene (33, 34); 4) a novel viral inhibitor of the cytokines GM-CSF and IL-2 (GIF)(35); and 5) chemokine binding protein (CBP).

The viral homologue of ovine VEGF is transcribed early in infection and is required for full virulence of the virus (36). The viral VEGF induces epithelial cell replication and induction of mitogenesis of vascular endothelial cells and increased vascular permeability (36). Loss of the viral VEGF results in decreased virus-induced blood vessel proliferation in the dermis at the site of infection (36). Induction of cellular proliferation and increased number of blood vessels present in the skin wounds is characteristic of the disease giving the lesions a raised appearance (7, 37). As wounds heal, virus-rich scabs form on the surface of the lesion. These scabs are shed from the

lesion at the end of the healing process thereby facilitating the spread of ORFV in the environment.

Not only does the VEGF cause the proliferation and repair of the damaged tissue, IL-10 also aids in the repair of these tissues (38). Mammalian interleukin 10 is an anti-inflammatory cytokine that is produced by monocytes and helper T cells (39, 40). IL-10 proliferation is important because it lets the body repair damaged tissue while limiting immune cell recruitment. The ORFV encodes a homologue to ovine IL-10. This viral IL-10 (virokine) is genetically, structurally, and functionally similar to ovine IL-10 (29, 41, 42). The vIL-10 is 80% similar to ovine IL-10, 67% similar to human IL-10, and 60% similar to caprine IL-10 (30). The vIL-10 is also functionally similar to ovine IL-10. In a murine thymocyte proliferation assay, the vIL-10 had similar dose-dependent effects on murine thymocytes as ovine IL-10 (29). This virokine downregulates the host inflammatory response and the production of antiviral compounds. Viral IL-10 is able to inhibit interleukin 8 and tumor necrosis factor alpha (TNF-a) production from ovine macrophages and keratinocytes and to inhibit IFN-γ and GM-CSF from ovine peripheral blood lymphocytes (30). Viral IL-10 also inhibits maturation, antigen presentation and migration of murine dendritic cells to inflamed skin (23, 43). A mutant virus strain lacking the vIL-10 used to challenge sheep induced higher interferon-γ levels in infected tissues than the parent virus (41). Lesions in sheep with the IL-10 knockout virus were smaller and less severe than lesions in sheep infected with the parent virus (44).

Immune evasion does not stop with the vIL-10 and VEGF. The ORFV blocks the stimulation of type I and type II interferons (45). Interferons are important for limiting

viral replication and, in humans, are regulated through MxA (type I interferons) and GBP1 (type II interferons). ORFV strongly inhibits both MxA and GBP1 in HeLa cells stimulated with interferon-α and interferon-γ (45). The ORFV encodes an interferon resistance gene that binds to double-stranded RNA (dsRNA). The production of the interferon prevents activation of PKR kinase and limiting host inhibition of virus replication (33).

The ORFV also encodes a novel viral gene that binds to and inhibits GM-CSF and IL-2 called GM-CSF inhibitory factor (34, 35, 46). The GIF protein is not found in any other poxvirus genera (35). GM-CSF is produced by a variety of immune cells including macrophages, T lymphocytes, natural killer cells, endothelial cells and fibroblasts. Its primary function is the stimulation and production of granulocytes, but it also acts to inhibit neutrophil migration and affects activation of macrophages. IL-2 activates the Janus kinase/signal transducer and activator of transcription, phosphoinositide 3 kinase/Akt/mammalian target of rapamycin, and mitogen-activated protein kinase/ extracellular signal-regulated kinase signaling pathways that are important for immune system activation and differentiation of T lymphocytes into effector and memory T-cells. Inactivation of GM-CSF and IL-2 by a viral protein decreases immune activation and allow propagation of the ORFV. Similar proteins have been found in other parapoxviruses including the bovine-specific pseudocowpox virus (35). The structural basis of GIF binding GM-CSF and IL-2 has recently been determined and GIF "employs a dimeric binding platform that sequesters two copies of its target cytokines with high affinity and slow dissociation kinetics" (47). In addition to

these novel mechanisms of evading the host immune response, ORFV also encodes a chemokine binding protein (CBP) that binds to human CC-chemokines, XCL1 (lymphotactin), and several CXCL chemokines (48-51). In sheep, the deletion of CBP results in attenuation of the virus with decreased lesion formation and lesion resolution at 5 to 6 days instead of 10 days required for the parent wild type virus (52). The tissues infected with the mutant virus harbored increased staining for major histocompatibility complex class II (MHC-II) suggesting the presence of greater numbers APC's as compared to tissues infected with the mutant virus complemented with CBP also called a revertant virus (52). This suggests that CBP may disrupt chemokine gradients and the migration of immune cells to the site of infection; however, tissues infected with the parent wild type virus had higher levels of MHC-II staining than the revertant virus despite the similar histopathological appearance of lesions in these two groups (52). Interestingly, the antibodies in all three groups were similar despite the significant difference in lesions between the CBP mutant virus as compared to the parent and revertant viruses (52).

Finally, the ORFV uses the CD 95 pathway to cause apoptosis in antigen-presenting cells (APCs) in a mouse model (53). In this study, mice were inoculated with ORFV intraperitoneally with ORFV, complete Freund's adjuvant (CFA), or a placebo (53). At six- and 12-hours post inoculation, mice were euthanized, and peritoneal cells were recovered by peritoneal lavage and lymph nodes were collected. Cells were then subjected to fluorescence-activated cell sorting (FACS) after being stained with antibodies against T lymphocyte markers including CD11b, CD8, CD4, CD69, and

CG45R/B220 (53). ORFV prevented T cell activation and induced apoptosis in monocytes and induced apoptosis through CD95 and the CD95 ligand (53). The authors hypothesized that the viral protein responsible for apoptosis is a structural part of the virus as both live and inactivated virus induced CD95 and apoptosis (53). The ability for the virus to induce apoptosis in the host macrophages and monocytes through a structural protein is remarkable and further study is warranted.

Immune evasion by the virus makes development of an effective vaccine against ORFV difficult. It is difficult to induce long lasting immunity to the virus as evidenced by the virus' ability to infect animals repeatedly. The viral IL-10 slows and inhibits the development of the memory immune response. A study of ORFV in humans shows that there is an immediate increase in peripheral mononuclear cells in infected individuals but there is a rapid decline shortly after this spike (54). This is the area in which the virus is the most interesting. It is a valuable tool to be propagate undetected by the immune system by silencing the immune response upon infection. There is significant economic cost to sheep and goat producers worldwide due to this virus and significant morbidity in affected animals. There is a need for a vaccine against the ORFV that provides long-lived immunity without using live virus that can potentially infect animal caregivers and contaminate the environment with viable virus.

The current vaccine on the market is ORFV scab harvested from the animals prior to its shedding. The scabs are ground and resuspended in a glycerol solution that is applied to an abrasion on the vaccinate (55). This method exposes the livestock worker to a zoonotic virus, and it potentiates the virus in the area where these animals are living.

There are also two other vaccines currently available outside of the United States that are cell culture derived vaccine. One of the major issues with these vaccines is that they do not induce long-term immunity and animals need to be revaccinated annually, if not more frequently, to ensure protection (7). Research is currently underway in the United States to produce a cell culture vaccine. The cell culture-based vaccine is much safer for the animal because, unlike the scab-based vaccine, it is free of bacteria that can cause secondary infections.

1.4. Uses of ORFV in vaccine production

The ORFV is being tested as a viral vector to produce vaccines against other viruses because of its immune modulating abilities and the self-limiting nature of orf infection. Two examples of this are, use of recombinant ORFV as vaccines against Borna Disease virus, and a vaccine against porcine epidemic diarrhea virus (PEDV). To protect neonatal piglets against PEDV passive immunity is a necessity (56). The ORFV contains a spike protein from PEDV to help immunize these neonatal piglets (56). Another example of orf virus as a viral vector is recent work to develop a potential vaccine against Borna disease virus (57). Borna disease virus is a neurotropic virus that infects horses, cattle, sheep, dogs, and other canids and may be infectious for humans. The virus causes neurological symptoms due to invasion of the brain by activated specific CD8 T cells and persists in the neural cells. In a recent study, a recombinant ORFV carrying a Borna virus protein induced protection in a rat model of Borna disease. The virus was cleared from the brain of immunized animals and 4 to 8 months after vaccination, animals were protected from the disease (57). These studies offer hope for

commercial development of an effective vaccine against Borna disease virus and suggest a possible use for ORFV based vaccines.

1.5. Summary

ORFV is an epitheliotropic Parapoxvirus that uses multiple proteins to evade the host immune response. Most of the central genes in the 138 kbp genome encode for propagation factors (58). The genome's terminal ends carry its important immunomodulatory genes that include VEGF, a viral homologue of IL-10, a chemokine binding protein, GM-CSF inhibitory factor and multiple hypothetical genes. VEGF increases epithelial cell replication and vascularization of the local area also allowing the virus to propagate in tissues. The viral IL-10, GIF, and CBP act locally and this local mediation assists in keeping the virus genome under the radar of the immune system. The reinfection of this virus is one factor that makes this virus stand out. The ability for the virus to do such an impressive job of hiding from the immune system that even after infection the animal can still be re-infected is purely remarkable. Designing an effective vaccine against ORFV, one able to activate the immune system enough to create memory cells and to prevent recurring infections, offers a significant challenge. It likely will require the virus to be used in tandem with an adjuvant that will stimulate immunity against the ORFV envelope or external proteins regardless if it is a modified live or inactivated virus. The unique interactions between the virus and the immune system that limit the infection to non-systemic disease make ORFV a promising viral vector for

future vaccines. The culture of ORFV is rather cumbersome, but with the constant discovery of novel culture methods this virus will be more frequently used in the future.

2. GENOME ANNOUNCEMENT AND ASSOCIATED WORK

2.1. Genome introduction

Orf Virus (ORFV) is a member of the Parapoxvirus genus that infects a scarified area of skin on sheep and goats typically around the mouth and nose (59, 60). ORFV has a morbidity rate around 89% in sheep and has had a substantial economic impact tracing back to 1787 (59, 61). This virus can cross skin or mucosal barriers that have been damaged and causes infections resulting in painful lesions that develop into scabs and eventually are shed from the body. The scab material is highly saturated with infectious ORFV and can remain infectious for 15 to 17 years (13, 20).

Sequencing the DNA of the commercially available, contagious ecthyma orf virus vaccine strain will provide the foundation for a future cell culture derived vaccine. The sequence reveals all the virulence factors and other immunogenic epitopes essential for vaccine efficacy. The genome needs to be similar enough to the other wild type ORFV that infect the sheep and goats that are candidates for this vaccine. A hybrid assembly approach was used to maximize the completeness and resolution of the genome. Nanopore, a long-read sequencing technology, was performed alongside the more accurate but short read Illumina technology.

2.2. Materials and Methods

2.2.1. Virus propagation and Extraction

The isolate sequenced here was harvested from an ovine testicular cell line that was infected with a commercially available, Ovine Ecthyma Vaccine Live Virus (USDA Product serial number: 1821.51). This is the first genome sequence obtained from this

commercial vaccine. Obtaining a complete genome of this strain of the virus will be an important reference for future vaccine production.

The vaccine was rehydrated in Hanks balanced salt solution and freeze thawed. The solution was filtered through a hydrophilic .45µm filter into a suspension of ovine testicular cells (ATCC OA3.Ts) supplemented with Dulbecco's Modified Eagles Medium. The tissue culture flask was then incubated 48 hrs at 37°C and refed with additional ovine testicular cells. Sterility of cell culture from bacterial and fungal contamination was screened at each passage and resulted in no detected contamination. The flask was harvested after an additional 48 hrs of incubation by repeat freeze-thaw cycles alternating between -80°C and 25°C. An additional passage of this virus was completed by the same method as before (co-infection) and harvested by freeze thaw. The final harvest was frozen and thawed two additional times to ensure the release of all viral DNA from host cells. DNA was extracted by DNeasy® Blood & Tissue Kit (Qiagen 69504) protocol "Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)" (62). The freeze thaw harvest material was then centrifuged at 4°C at 2000 x G for 10min. The supernatant was collected and 600 µL was used for extraction (in place of resuspending cells in PBS) and 60 µL of Proteinase K solution reagent was added. Then, 600 µL of Buffer AL was added and vortexed. The mixture was incubated at 56°C for 10 min. After incubation, 600 µL ethanol was added to the sample mix, followed by vortexing. The mixture was then added to a spin column and centrifuged according to the manufacturer's recommendation. This step was repeated three times in

the same tube discarding flow-through each time. The manufacturers protocol was followed, except for the elution step when 50µL was used in place of 100 µL.

2.2.2. Library Preparation and Sequencing

The quality of the genomic DNA was verified on a genomic DNA TapeStation run (Agilent) prior to sequencing and quantified using the Life Technologies Qubit high sensitivity dsDNA assay. For all following programs and bioinformatics, the default parameters were used except where otherwise noted.

Illumina libraries were prepared by hand using the Illumina Nextera DNA Flex Library Preparation Kit following the manufacturer's protocol, with samples normalized to 100 ng of DNA. DNA was sequenced on an Illumina MiSeq using the 2x300 v3 sequencing kit. All data were uploaded to Illumina's cloud-based resource, Basespace, for run monitoring, FASTQ generation, demultiplexing and adapter trimming. The resultant sequencing output of paired end read sets contained approximately 1.7 million reads of 301 bp. Using Trimmomatic, reads below 200bp were filtered and the remaining reads were trimmed based on sliding window of 5, with bases removed if the average quality dropped below Q20 within the sliding windows (63). This resulted in 1.4 million retained reads.

Nanopore libraries were prepared following the manufacturer's protocol for 1D PCR Barcoding of Genomic DNA using the Nanopore SQK-RAD004 Rapid Sequencing Kit. Genomic DNA quality was verified on a genomic DNA TapeStation run (Agilent) confirming high quality (>60 kb). DNA samples were normalized and sheared with a

Covaris g-Tube to generate ~8kb DNA fragments. Sample was dA-tailed and adapters ligated to the fragments before a specific barcode was added by PCR amplification using the NEG LongAMP Taq 2x Master Mix (1 cycle denature 3 min at 95°C; 13 cycles denature 15 s at 95°C, anneal 15 seconds at 62°C, extension 10 min at 65°C, and final extension 10 min at 65°C). The prepared sample was loaded onto the MinION Flow Cell for sequencing. Sequencing data collection and base calling was performed by the MinKNOW software with real time base calling enabled. Following base calling, the run resulted in an output of read sets containing 1.6 Gbp of data. Reads were corrected and trimmed using Canu Version 1.8, with a minimum length of 500bp (64).

2.2.3. Sequence Assembly

After sequencing, a total of 1.4 million Illumina paired-end reads were checked for host (*Ovis aries*) and contaminating (bacteria) reads, which were filtered out by using the Texas A&M Institute for Genome Sciences and Society's (TIGSS) Virus Identification Pipeline. The pipeline was originally developed at the institute to identify viral contamination in samples through next-generation sequencing. In brief, the paired-end reads were first combined and mapped to the host genome assembly using bowtie2 in local mapping mode (65, 66). The standard protocol for the program is to remove any bacterial reads by mapping to GOTTCHA bacterial signature database at species level (67), following which the final 31,626 filtered paired-end viral reads were deinterleaved and assembled using Unicycler Version 0.4.7, resulting in a 10 contig assembly of 124,608 bases.

The Nanopore reads were mapped to the Illumina assembly using minimap2 Version 2.14 resulting in 27,573 retained reads. The mapped long reads, and filtered paired-end Illumina reads were combined provided a minimum of 164X coverage of the orf genome, and were assembled together using Unicycler in conservative mode to form a hybrid assembly of 7 contigs with a total of 165,738 base pairs. The longest contig contained 131,930 bp. Simultaneously, the Nanopore reads were assembled using Canu to see if the long read only assembly would be more complete. While this assembly would be less accurate, the Illumina reads could be used to polish the assembly using Pilon, which results in a highly accurate and often more complete assembly. Unfortunately, the Nanopore Canu assembly resulted in 126 contigs and was discarded, as the Unicycler hybrid assembly was more complete. All assembly to this point was de novo and no reference genomes were used.

2.2.4. Genome Hybrid Assembly

The Unicycler hybrid assembly was run through ragout using the orf virus OV-IA82 Genome from GenBank accession number AY386263:1 as a reference in order to attempt to scaffold the contigs correctly (68). This resulted in a single scaffolded assembly containing 2 contigs, with 5 unplaced contigs. These unplaced contigs were examined using BLAST and aligned to other orf viruses in GenBank. Three were found to be lingering host cell contamination and were consequently discarded. The two other contigs in the unplaced data were overlapping contigs duplicated in the single scaffold assembly and were similarly discarded, resulting in a final, single scaffold, 2 contig assembly. To confirm contamination and contig validity BLAST data was run through

NIAID Virus Pathogen Database and Analysis Resource (ViPR) (69). NCBI BLAST confirmed valid contigs as well as overlapping and irrelevant contigs.

2.3. Results

The draft genome was assembled and resulted in a mostly complete genome. The hybrid assembly was a single scaffold, 2 contig assembly with 134,882 bp and a GC content of 64.14%. The contigs were 101,329 bp and 33,553 bp in length. The gap between the two contigs is a location that includes much of the diversity between this genome and the other ORFV genomes published. This gap is evident when run through the NCBI BLAST program, which demonstrates it is 97% complete compared to the complete orf genome OV-IA82 (GenBank AY386263.1) and similar complete genomes.

Initially, only Illumina sequencing was performed, resulting in highly accurate MiSeq data, but there was significant host cell contamination resulting in an assembly of 75,000 contigs using the SPAdes algorithm (70). While these reads were being filtered, the Nanopore sequencing technology was included to help close the genome with its much longer, yet less accurate reads. The two sets of data were combined in a hybrid assembly to optimize the accuracy of the short reads with the completeness of the long reads to form a near-complete, highly accurate genome.

The de novo assembly was completed using Unicycler, resulting in a hybrid assembly that was the most accurate and complete when compared to assembling the genome with either of the sequencing technologies separately. The hybrid assembly contained 7 contigs that were scaffolded and the unplaced contigs analyzed and subsequently discarded, giving a result of a 2-contig, single scaffold draft genome. This

draft genome was published to GenBank under the Bioproject PRJNA563624. The raw reads were deposited into the SRA under accession number SRR10102957 (Nanopore fast5 files) and SRR10102956 (Illumina fastq). The genome is available under GenBank MN454854.

Statistical Analysis of Genome

There are 13 ORFV genomes available through GenBank for comparison. One of the most similar genomes was used in this project as a reference genome (OV-IA82 GenBank AY386263:1). The other genome that is noted as a primary reference in the NCBI library is OV-SA00 GenBank AY386264, which was collected from San Angelo, Texas, the same city that the ORFV strain described in this study was collected (8). The Parapox genus is an ancient virus group and it is well known for the genetic conservation within virus species. Of the 13 complete genomes all have >97% identity to the genome being discussed.

The draft genome is 134,893 bp in length. The reference genome OV-IA82 is 98.71% similar to the hybrid genome assembly according to the NCBI BLAST program. The Genome Annotation Transfer Utility (GATU) program from the Virus Pathogen Research database (ViPR) was run with reference strain OV-IA82 GenBank AY386263:1 and showed the final assembly to be very similar to the reference genome, apart from 4 genes. Two of the 4 genes were Extracellular Enveloped Virus (EEV) glycoproteins and were 67.7% and 66.9% similar to reference genome. These glycoproteins are present on the surface of infected cells and are not required for viral replication in tissue culture (71). The NCBI BLAST output also shows that the two EEV

glycoproteins are overlapping the section that is missing from the hybrid genome data, which may account for the lack of similarity. Another of the 4 proteins is one of the two A type inclusion proteins, which was 69.3% similar to reference genome. A type inclusion proteins are believed to protect infectious virus particles after release into the environment (72). The final dissimilar protein was a hypothetical gene that was not included in the hybrid genome.

There are four virulence factors that are present in the ORFV genome. Vascular endothelial growth factor increases vascularity and scab production as well as increased nutrient availability for infected area. Amino acid similarity to other published genomes as follows: OV-IA82 81.4%, OV-SA00 52.6%, OV-HN3/12 96.2%, and OV-NZ2 99.6% similar to hybrid genome. The virulence factor vIL-10 is a chemokine produced by the virus that downregulates the immune response. Amino acid similarity to other published genomes as follows: OV-IA82 100%, OV-SA00 94.1%, OV-HN3/12 96.2%, and OV-NZ2 95.2% similar to hybrid genome. GIF is found to bind to granulocyte-macrophage colony-stimulating factor and interlukin-2 which reduces the upregulation of immune response to the infected area. Amino acid similarity to other published genomes as follows: OV-IA82 99.6%, OV-SA00 97.4%, OV-HN3/12 98.5%, and OV-NZ2 99.6% similar to hybrid genome. Chemokine binding protein binds chemokines with high affinity and prevents them from interacting with immune cells to upregulate immune response. Amino acid similarity to other published genomes as follows: OV-IA82 87.1%, OV-SA00 87.4%, OV-HN3/12 90.3%, and OV-NZ2 90% similar to hybrid genome. These virulence factors play a major role in the overall success of ORFV

survival. This genetic similarity shows that the virus could be a good virus for vaccine production.

2.4. Discussion

Host cell DNA posed a major problem when assembling the hybrid genome. The initial Illumina sequencing contained 1.7 million reads, of which only approximately 37,000 belonged to ORFV after filtering. Similarly, Nanopore sequencing initially produced 1.6 Gbp of data and, after mapping, only approximately 27,000 reads belonged to ORFV. While there was enough coverage to assemble an accurate and almost complete genome, had there been less host contamination it is likely that the genome would have been fully resolved even with only the Illumina sequencing. The large genome associated with *Ovis aries* accounted for most of the host contaminant. The only steps used in this study to reduce host cell DNA contamination was separation of host cell material via centrifugation after freeze thaw. Freezing mammalian cells even at -80°C forms crystals that cause cell lysis resulting in release of host cell DNA. The centrifugation cannot occur before freeze thaw due to the same reason. The cell lysis must occur to release the viral particles from the cell cytoplasm. One method that could have been used to isolate the viral DNA was electrophoretic separation of the ORFV DNA from host cell DNA(73). The DNA of each organism, host cell and virus, have different molecular weights; ORFV DNA is 88.8 x 10^6 whereas *Ovis aries* is much larger (74). The viral DNA will move much faster through the agar than the sheep DNA. The separated DNA can then be removed from the agar and sequenced now that it is pure ORFV DNA. DNA purifying steps such as these should be used in order to maximize

the genome coverage gained through whole genome sequencing and increase the likelihood of complete and accurate genomes, without requiring additional decontamination steps downstream.

3. CONCLUSIONS

3.1. Overarching importance

ORFV is a significant pathogen infecting animals in the sheep and goat industry as well as the humans who interact with those animals. The fact that there are only outdated and inefficient vaccines available in the United States is a problem for controlling Contagious Ecthyma in America. Most vaccines currently available are prepared in such a way that they potentially infect animals not only with ORFV, but also with other bacterial and viral pathogens that were contained in the infectious scab material utilized for the vaccine. There are multiple benefits of having a cell culture vaccine, including the fact that the virus in the vaccine is more pure than the wild type found in the scab vaccine, and less likely to contain other viral or bacterial pathogens. The ORFV published here shows the foundation of the vaccine that is under development. There are only 13 complete genomes available through the NCBI website. The limited amount of complete ORFV genomes available makes it that much more important for more data to be available about this virus. The ORFV usually has 130 putative genes defined in the genome consistent with the genome sequenced here.

3.2. Comparison to other ORFV

The hybrid assembly was completed *de novo*. The importance is that there was no bias by the researchers or programs assembling this genome. The primary goal of the analysis and sequence of this genome is to publish the viral strain present in this commercial vaccine and define any major differences in this strain from others. Of the 13 genomes available for analysis, one of the most similar genomes was used in this

project as a reference genome (OV-IA82 GenBank AY386263:1). The other genome that is noted as a primary reference in the NCBI library is OV-SA00 GenBank AY386264, which was collected from San Angelo, Texas, the same city that the ORFV strain described in this study was collected (8). The Parapoxvirus genus is an ancient virus group and it is well known for the genetic conservation within virus species. Of the 13 complete genomes all have >97% identity to the genome being discussed.

The draft genome is 134,893 bp in length. The reference genome OV-IA82 is 98.71% similar the hybrid genome assembly according to the NCBI BLAST program. The GATU program from ViPR was run with reference strain OV-IA82 GenBank AY386263:1 and showed the final assembly to be very similar to the reference genome, apart from 4 genes. Two of the 4 genes were EEV glycoproteins and were 67.7% and 66.9% similar to reference genome. These glycoproteins are present on the surface of infected cells and are not required for viral replication in tissue culture (71). The NCBI BLAST output also shows that the two EEV glycoproteins are overlapping the section that is missing from the hybrid genome data, which may account for the lack of similarity. Another of the 4 proteins is one of the two A type inclusion proteins, which was 69.3% similar to reference genome. A type inclusion proteins are believed to protect infectious virus particles after release into the environment (72). The final dissimilar protein was a hypothetical gene that was not included in the hybrid genome.

Of the 130 annotated genes in the ORFV genome there are three that are of more interest for vaccine production. The conserved genes represented in the genome include the viral IL-10, VEGF, and ankyrin repeat domain-containing protein M-T5 (ANK). All

three of these proteins are virulence factors for the ORFV. Viral IL-10 decreases immune response through mimicking host IL-10 production. VEGF is important in increasing vascular growth and nutrients transport for production of ORFV in infected cells. The VEGF is a potent angiogenic factor for the surrounding tissues of the infected cells (75). The angiogenesis factor that the VEGF produces is also found in tumor production as, similar to the high nutritional needs of a tumor, ORFV requires an increase in nutritional supply and utilizes VEGF to achieve it. The third protein, ANK, is one not previously discussed in this paper. ANK plays a critical role in ensuring the infected cells are reprogramed to produce ORFV in the hijacked cell (76). All viruses are obligate intracellular parasites and, in the case of the ORFV, the host cell is hijacked and turned into an ORFV manufacturing machine that disregards its normal cellular functions. These three proteins ensure the cell has the protection it needs, nutrients it needs, and that it is reprogrammed to produce more infectious virus particles to introduce back into the environment. There are also additional proteins included in this genome, such as the A type inclusion protein that plays a role in ensuring mature virus particles remain infectious for long periods of time (72).

3.3. Sequence Data Use in Industry

Cell culture vaccine production produces a consistent and pure product. The viral component of the vaccine is standardized by using a $TCID_{50}$ calculation to measure the amount of infectious viral particles in a volume of fluid. This protocol is available from the United States Department of Agriculture division Center for Veterinary Biologics Supplemental Assay Methods. The use of a virus propagated in cell culture can attenuate

the virus, especially if serum or the cell line is from the lineage of the typical host of the virus. The use of an attenuated virus (such the genetic modification of the EEV gene and some other minor changes) can limit the possibility of environmental contamination with virulent virus.

As ORFV produces a long-lived immunological response, the vaccine will most likely be a Modified Live Vaccine and using an attenuated virus will reduce potential side effects. The ORFV genome discussed here is a solid foundation for defining the virus being used in production. The data will be useful when analyzing production methods in that the virus can be passaged many times in cell culture. Each generation in culture increases genetic shift and variability. The virus can be sequenced again at the maximum amount of passages or vial generations. The ultimate goal is to have a vaccine that has a long-lived immune lifespan with minimal hazard to the animals being immunized and the animal handlers vaccinating and handling these animals.

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