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RESEARCH

In process metagenomic analysis of LSDV harvest for quality control

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ABSTRACT

Background: Vaccination against lumpy skin disease is carried out by heterologous sheep pox vaccine (SPPV) giving un-satisfiable results and incomplete protection in vaccinated cattle, since that it was a must to produce a homologues vaccine for LSDV.

Methods: A local isolate of LSDV strain was propagated on MDBK cell line and CPE was appeared after 72 hours as cell rounding, cell aggregation and cell degeneration with titer of $10^{5.5}TCID_{50}$/ml, DNA extraction and PCR for the candidate strain was performed using universal and specific primers for virus identification and confirmation. The size of the produced amplicons was 192 pb for the universal primer and 502pb, 1452pb for the specific primers respectively. Metagenomics is done to evaluate a virus harvest for standardization by applying a suite of genomic technologies and bioinformatics tools to directly represent the genetic content of entire communities of organisms in the given harvest.

Results: In this study a 3rd generation sequencing (TGS) was applied using a Nanopore technology (MinION) based on identification of DNA bases by measuring the changes in electrical conductivity generated as DNA strands pass through a biological pore (nanopore). Its portability, affordability, and speed in data production makes it suitable for real-time applications, the release of the long read sequencer MinION has thus generated much excitement by producing a high microbial diversity in biological samples that was limited by using second generation sequencing as Illumina . DNA concentration and sequencing had been performed in demands for faster and more accurate methods to analyze, characterize and qualify the virus as a seed for future vaccine production. Analysis workflow starts on a high performance laptop device (GENUS), sequence data is generated for bacteria, viruses, fungi and archaea that present in the sample it was then classified to subspecies and strain level in a quantitative manner in approximately 48min and 23 second.

Conclusion: This method allows screening all the virus harvest content in non-selective, non- targeted manner. The reasons to choose this technology in qualification of the TC propagated LSDV as a one-step quality control for in process LSDV indicating all what in the sample as well as an indication for virus purity. The presence of LSDV with very high degree of confidence and the presence of other similar species in the capripox genus as they share a lot of conserved areas all over their genomes remain to be addressed to fully exploit the potential of the nanopore technology, together with some other conventional testing before judging the sample.

Keywords: Vaccination, LSDV, 3rd generation sequencing, Nanopore.

BACKGROUND

Lumpy skin disease is an infectious, eruptive, occasionally fatal disease of cattle, and is economically important (*Davies, 1991; Coetzer et al., 1994; Fenner, 1996*),it belongs to genus Capripoxvirus which comprises LSDV, sheep pox virus (SPPV) and goat pox virus (GTPV). The prototype of LSDV, Neethling strain, was isolated for the first time in South Africa (*Alexander et al., 1957*). The viral genome is 150,000 bp long, double-stranded DNA, covalently cross-linked at the ends, similarly to other poxviruses, it contains 156 ORFs or putative genes. The genes encoding host range, virulence and immune evasions are located at the terminal parts of the genome *(Tulman et al., 2001*) .Vaccination in endemic areas is the only recommended tool

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for virus control together with biosecurity, vector control, quarantine and animal movement control. *(Eva 2014*)Although It is widely agreed that vaccination using a homologous vaccine is the only effective way to control the spread of LSDV in such endemic countries (*EFSA Journal 2015*), but in central and northern Africa and in the Middle East, where the distribution of SPP, GTP and LSD overlap, attenuated SPPV vaccines, such as KSGP O-240, Yugoslavian RM65 and Romanian SPPV strains, have been used against LSDV (*Brenner et al., 2009;Davies, 1991; Kitching, 1986; Somasundaram, 2011*.) Current vaccination in Egypt is carried out by Romanian (SPV) attenuated sheep-pox vaccine for cattle against LSDV .Recently we have been affected by a wave of sever overlapping LSDV outbreaks indicating incomplete protection by heterologous SPV vaccine in cattle (Eva .T *et al;*2014) For this reason it was important to produce a specific homologues LSDV vaccine that should be investigated by complete genomic and molecular study to claim and assure a standard with a quality control step of LSDV harvest for future vaccine production in Egypt .

Vaccine production requires the use of complex production methods, meticulous quality control steps that ensure the products effectiveness at their point of use. In process quality control are of particular importance in the manufacture of veterinary vaccines. Viral metagenomics, which involves viral purification and shotgun sequencing, (*Chapman et al. 2004*) has proven to be one of the most important tools for understanding viral diversity and describing novel viruses in new diseases and scanning for the existing ones for any changes or mutations (Alavand S.V *et al;* 2012) it can simplify the in process quality control by identify very small amounts of mutant viruses in live viral vaccine preparations, and enables accurate quantification as what previously used in deep sequencing used in Oral poliovirus vaccine for evaluation of the virus mutations that is primarily preformed on culture-enriched isolates(*Malaya K.et al;2010*). It also allows for the rapid generation of very large amounts of sequence data (*Neverov et al;2010, Schirmer et al; 2015*). Anew concept about in process quality control during veterinary vaccine production has been recently described using viral metagenomics due to the advancements in sequencing technology and development of bioinformatics tools for nucleic acid sequence assembly and annotation, information on novel viruses and diversity of viruses (*Alavand S.Vet al;2012*), this tool has been also recently used to reveal the vast majority of microbial biodiversity that had been missed by cultivation-based methods(traditional quality control methods) which is usually directed giving unfair judgment. (*Naccache S.N, et al.2014, Schuster S.C; 2008*) Metagenomics offers a powerful lens for viewing the microbial world that has the potential to revolutionize understanding the suitability and validate viral seeds and it's culture during vaccine production ,there exists a great potential with viral metagenomics for discovering un targeted microorganisms and allows for unbiased detection of virtually any pathogen present in a given sample (*Victoria JG et al;2009– okili JLet al; 2012)* even the under-represented microbial taxa in culture-dependent investigations(*Arwyn Edwards et al;2017*). Additionally, viral metagenomics can help our understanding of viruses situation in tissue culture and it's variation during propagation (*Neverov et al;2010, Schirmer et al; 2015*) and the formation of many genetically related viral variants referred to as quasi-species that represents the substrate for the vaccines and antiviral drugs*(Capobianchi M. R. et al;2012).*

In this study we used a low-cost, portable and direct sequencing MinION Nanopore platform that can be used for characterization as well as metagenomic analysis that offers a higher accuracy and a grater throughput with very long reads in real time data (*Jain M et al; 2015*) that allows identification of more species than short-read method data, that is very difficult to establish if similar genes are present in the same organism. We can connect the genes if they are found on a single DNA molecule with long sequencing reads, provided by third generation sequencing technologies*(Caner Bağcı et al;2019*) Also it facilitate accurate classification of the

microbial community composition in a given sample (*Nowak.M. et al ;1992*), (*Jasmijn et al;2017*) A direct DNA sequencing was performed on an array of nanopores, as developed by Oxford Nanopore Technologies (ONT)(*Adrian Viehwegeret al;2019*)on a tissue culture propagated and attenuated local Egyptian strain of LSDV harvest

MATERIALS AND METHODS

Virus and cells:

The virus was previously collected from locally isolated LSD virus from Ismailia outbreak 1988 of cattle origin the local isolate of LSDV (Ismailia 88 strain) is kindly obtained from Pox Vaccine Production and Research Department, Veterinary Serum and Vaccine Research Institute Abbasia, Cairo, Egypt. Each ampoule had A titer of log10 $^{5.5}$ TCID50 /ml. The 80th passage of LSD virus was used 10 times propagation on MDBK cell line **(*Aboul soud, 1999)***.the virus infectivity titers was calculated according to **(reed *and muench, 1938*).**

PCR:

DNA extraction:

DNA was extracted from virus suspension using Genomic mini kit ***(Gene aid)*** following the manufacture's instruction.

Identification using universal Capri pox primer *(Ireland & Binepal, 1998)*:

Table 1: Sequence of forward and reverse primers used for amplification of viral attachment protein encoding gene (***Ireland & Binepal, 1998):***

	Sequence
Forward primer	(5’ TTTCCTGATTTTTCTTACTAT 3’)
Reverse primer	(5’AAATTATATACGTAAATAAC 3’)

Table 2: Cycling conditions of the universal capri pox primer:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Viral attachment protein	95°c 1 min	94°c 30sec	58°c 30sec	72°c 70sec	36	72°c 5 min

Confirmation of LSDV using specific primers:

Were supplied by Metabion (Germany) that are specific and amplify a specific part of the genome .LSDVF-Aus ,LSDVRo-Aus primers with high detection sensitivity and LSDVRi-AUS amplicon ***(Ausama A.A et al;2012***) as shown in table(3)

Table 3: LSDV specific primers

Primer name	**Primer sequence**	**Location**
LSDVF-Aus	(5`-GTAATTGTTTGTTAAGTAATTAATC-3`)	94600 - 64624
LSDVRo-Aus	(5`-GTCCAAGCTAATGCATATCGGAC-3`)	96160 -96182
LSDVRi-Aus	(5`-GTTATATCAAGATTTGATTTCCG-3`)	95007 -95103

Table 4: Thermal Cycling conditions for LSDVRo-Aus primers during PCR:

Primary denaturation	Secondary denaturation	Annealing	Extension	No.of cycles	Final extension
94°c 4 min	94°c 1 min	50°c 30 sec	72°c 30 sec	35	72 °c 10 min

Table 5: Thermal cycling conditions for LSDVF-Aus and LSDVRi-AUS primers during PCR:

Primary denaturation	Secondary denaturation	Secondary denaturation	Extension	No.of cycles	Final extension
95°c 3 min	95°c 30 sec	51 °c 30 sec	72°c 1.5 min	42	72°c 10 min

Ultracentrifugation for virus concentration:

Ultracentrifugation of 200ml virus harvests every 50ml in a separate cup at 10,000 rpm for 1 hour for virus concentration; the pellet was suspended in 2ml of nuclease free water.

MinION nanopore sequencing:

DNA extraction for sequencing

DNA was extracted from virus harvest after 3 cycles of freezing and thawing using phenol chloroform extraction method after lysis and ethanol perception *(Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983.)* the pellet was suspended in 10 µl of nuclease free water.

Quality control:

- Kits and flow cell were stored at +4°C and were surrounded by refrigerated cold pack until use.
- DNA was quantified using Qubit 2.0 fluorometer ds DNA High Sensitivity assay kit *(Thermofisher, USA)* following manufacturer's protocols.

Barcoding:

The Rapid Barcoding Kit (SQK-RBK004) generates barcoded sequencing libraries from extracted DNA in 10 minutes using a simple 2-step protocol. At the heart of the kit is a transposes which simultaneously cleaves template molecules and attaches barcoded tags to the cleaved ends there are 12 unique barcoded tags in the kit. Barcoded samples are pooled and Rapid Sequencing adaptors are then added to the tagged ends. Adaptors are attached by ligation. Kit is optimized for simplicity and speed, rather than for obtaining maximum throughput. Due to the simple nature of the workflow and the fact that little sample manipulation is required (e.g. minimal pipetting steps and no clean-ups), some very long reads can be achieved with this kit despite the required transposes fragmentation. The Rapid Barcoding Kit recommends a total input of 400 ng DNA, and is optimized for samples which contain long fragments (>30 kb). Addition of less than 400 ng , or shorter fragments could compromise sequencing throughput and read length.

Finally 10µl of pooled sample is transferred into clean DNA LOBind tube.

Library Preparation:

MinION sequencing libraries were produced using the ONT 1D Genomic DNA ligation *(SQK-RBK004)* protocol. The library was purified using Agencourt AMPure XP beads *(Beckman Coulter Inc., CA, and USA).* The amount of recovered DNA was quantified using a Qubit fluorometer and a 400ng genomic DNA with 5µl of internal control DNA, and finally 11µl of DNA library, 34µl Sequencing Buffer (SQB), 25.5 µl Loading Beads, 4.5 µl Nuclease-free

water. A total 50 µl of sample is added to flow cell via Spot ON sample port, and 200 µl of priming mix into flow cell via Priming Port. Process for library preparation using the (SQK-RBK004) Sequencing Kit (ONT) is preformed according to the manufacturer's instructions.

Sequencing and base calling (rev.3.9.3):

Sequencing was performed using Flow cell No: R.4.1 FLO-MIN106. ONT fast5 data files were base-called using the ***ONT Albacore*** module. Base calling is such a very compute intensive operation; we should only do this on a subset of files depending on different computational demands.

Analysis of MinION Data:

Local base calling was performed using MinKNOW automatically in real time. 1D2 reads were generated from FAST5 reads by Albacore **(V 2.2.1).**

RESULTS

Virus propagation on MDBK cell line:

Appearance of cytopathic effect of the virus on the cells that is characterized by cell rounding and cell aggregation that scattered all over 50% of the monolayer sheet within 72 hours post inoculation and increased gradually till cell detached.

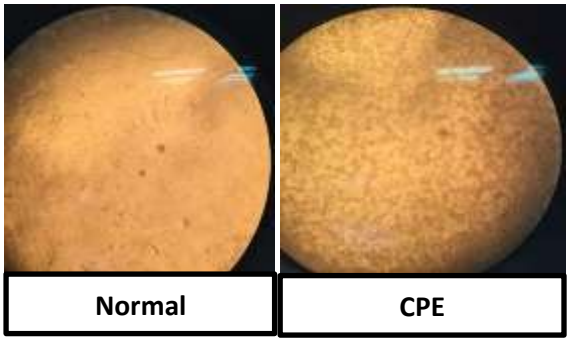


Fig. 1: CPE of the virus compared to normal cells with 4 x magnification powers 4 days post inoculation.

Virus titration:

Titration of LSDV was carried in MDBK cell line the titer was $10^{5.5}$ TCID50/ml.

PCR identification using universal capripox primer *(Ireland & Binepal, 1998)*:

The size of PCR product of tissue culture propagated LSDV(local strain) fragment using universal capripox primer was ~192pb as shown in figure(**2**).

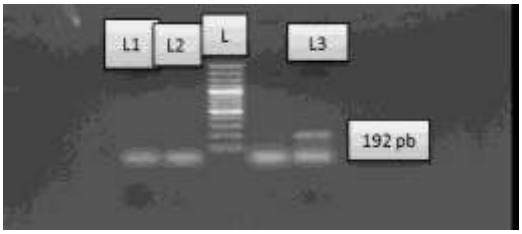


Fig. 2: specific product of capripox virus at the correct expected size of ~192pb using universal capri pox primer *(Ireland & Binepal ; 1998)* L: DNA ladder (high molecular weight nucleic acid marker).L1: negative control,L2: nuclease free water,L3: positive sample LSDV (local strain)

PCR conformation using specific primer for PCR:

The size of PCR product of lumpy skin disease virus (local strain) fragment of the LSDVF-Aus, LSDV-RiAus produce a 502 pb amplicon as shown in figure (3).

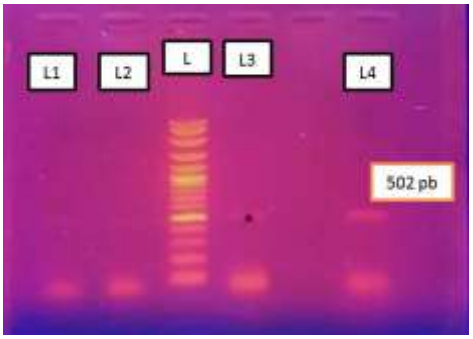


Fig. 3: specific PCR product of lumpy skin disease at the correct expected size (~502pb) :L: DNA ladder (high molecular weight nucleic acid marker),L1: sheep pox virus (negative control),L2: nuclease free water (negative control),L3: LSDV with dilution 10^{-3},L4: LDSV with dilution 10^{-4}

PCR confirmation using the high specific LSDV primer:

The size of the PCR product of the local strain using LSDVRo-AUS primer was **1452** as shown in figure (4)

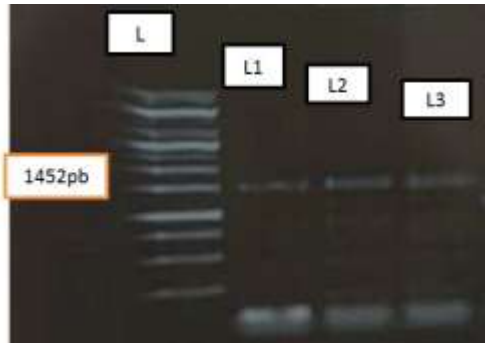


Fig. 4:L1, L2, L3: positive samples,L: DNA ladder

Result of "What's in my pot" analysis (WIMP) (rev.3.2.3):

The analysis shows reads analyzed as a total number of reads processed by WIMP and reads classified as a number of reads that could be classified due to non-contiguous perfect matching alignments against a set of references and reads unclassified as a number of reads for which no reliable classification was available shown in figure(**5**).

Fig 5:

Read unclassified	8,517
Read classified	3,521
Read analyzed	12,038

To allow straightforward interpretation of WIMP results an interactive report is created which allows the user to change several display features in taxa classification rank as family, genus and species shown in figure (**6).**

Fig 6: it reveals the taxa classification at genus level with 2,571of the reads are capripox genus including sheep pox ,goat pox and lumpy skin disease viruses, it also include a large number of reads as a homo-sabins due to propagation of the virus on mammalian cell line
(Madin derby bovine kidney cells-MDBK**)**

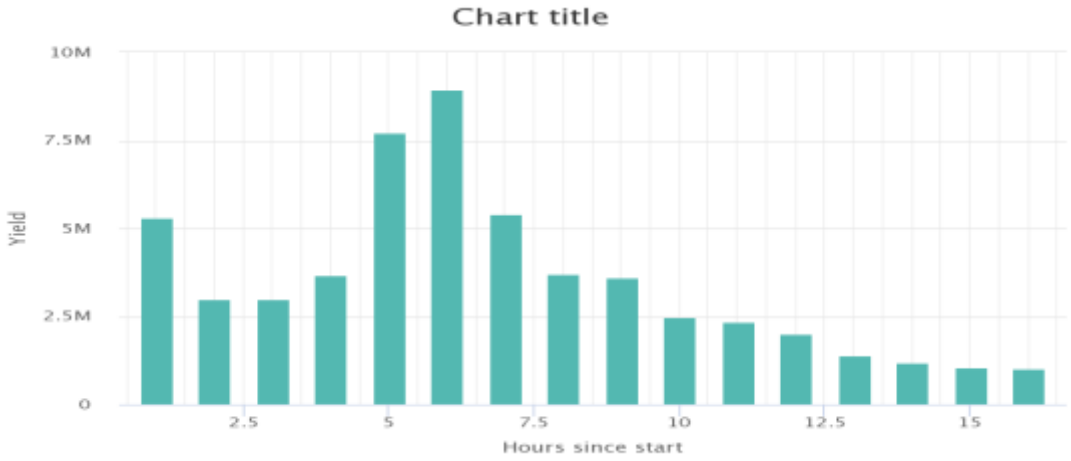
Fig. 7: Shows the taxa classification at a higher specificity rank which is the species showing LSDV as 1,417 cumulative reads with goat and sheep pox as 233 and 187 cumulative read respectively the presence of the other capripox species goat pox and sheep pox DNA with very high confidence is because all of the three species shared a conserved genomic areas in genus capripox and cross immunity is then effective due to this genetic similarity (Kitching;1983).The relatively low confidence of the other species may indicates that none of them is in the

database, whereas the high confidence at the higher subspecies node indicates the presence of Lumpy skin disease in the database detected as spiked-in that matches closely the added amount, by mass (3.5% of total DNA).

Sample composition: is a donut chart showing the relative proportions of reads from the species found in the sample, and the calculated confidence level. The area of the donut segment for each species is proportional to the read count. The color of the arc corresponds to the confidence in the Classification score section as shown in figure (**8**):

Fig. 8: Sample composition: The circle shows the percentage of reads for each superkingdom (virus and eukaryota only) it shows 65% for viruses and 35%for the Eukaryota due to the presence of the homo-sabians from MDBK cells . the bactrial load of 1%would suggest the presence of their DNA instead of the living organism in an inactivated form .

The chart shows time taken from beginning of sequencing and yeild of bases that is recognized as read count shown in fig (**9**):



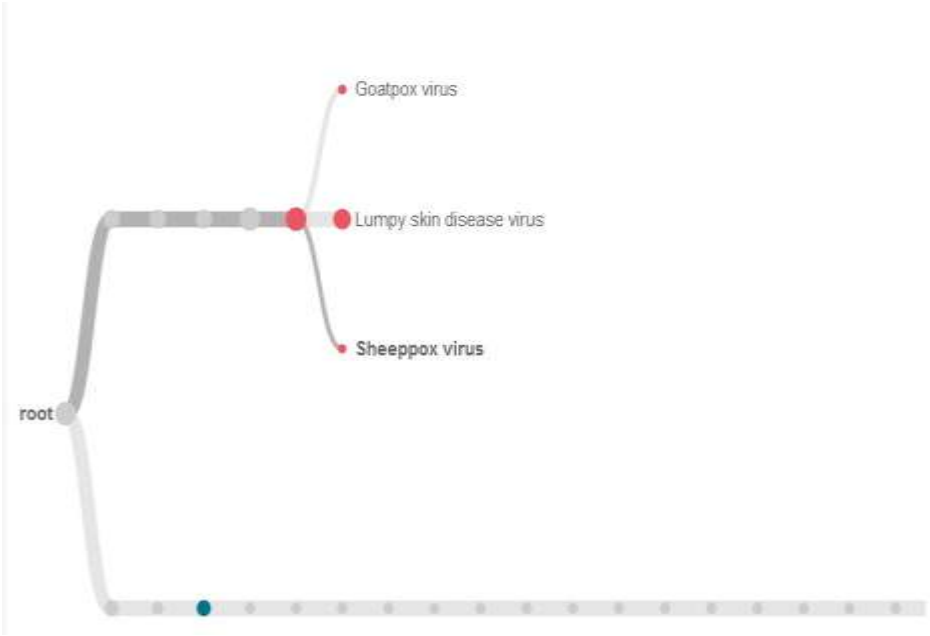


Fig. 10: Metrichor WIMP application report for species present in LSDV harvest (LSDV used as aspike -in): This figure shows the results from local basecalling carried out in MinKNOW or using the Albacore software showing the speed of the sequencing in obtaining large amount of data bases yeild per hours with a total yeild of 7.6Mbases and average sequence length 630 bases.

Subtree representing the NCBI taxonomy associated to the most common assignments listed in fig(7), thickness of the branches is proportional to the count reads along the lineage.
Finally the WIMP shows a work flow success 100 % and a mean quality scores of 9.67(the higher this classification score, the more confident that the classification was correct) (Sissel Juul *et al*;2015) as shown in figure (11,12)respectively.

Fig. 11: The work flow success is a demonstration of the over all run including all the barcodes .

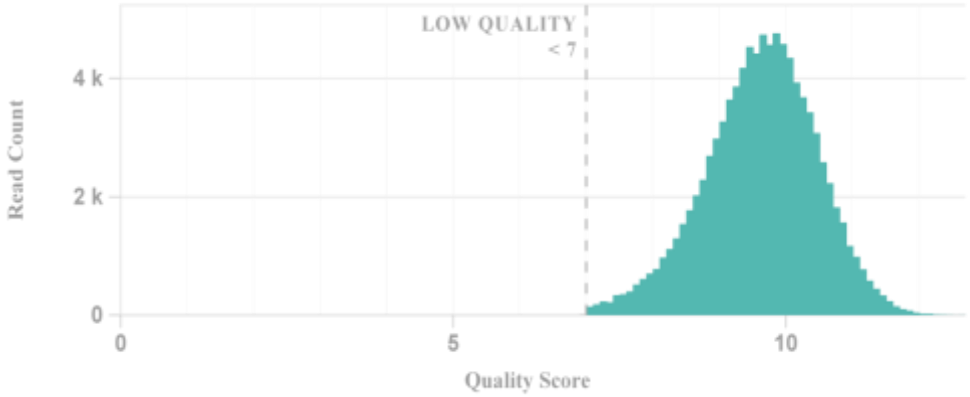


Fig. 12: The quality score shows a higher confidence in the classifiaction of the taxa for the input DNA sample.

DISCUSSION

Lumpy skin disease virus(LSDV) was isolated for the first time from diseased cattle in Egypt in 2 disease outbreaks at 1988(*House ;1990*).vaccination is the most effective antiviral interposition which mobilizes the host's immune system to protect animals from viral infections and Only live attenuated SPV vaccines are currently the available vaccine giving incomplete protection for cattle against LSDV*(Eeva et al;2014*), also many over lapping out breaks that occurring through the last years with a large debate for the cause and the reason of the problem and since that the preparation of new vaccine especially homologues ones as live attenuated LSDV vaccine would be a target that will provide 100% immunity to every individual animal(*Paolo Calistri et al ;2019*), these vaccines provide good protection if sufficient herd coverage is achieved (over 80–90%) and if maintained by annual boosters (*Kitching; 2003*), so it is the most effective way For sustainable effective protection and prevention of further spreading of LSDV. Molecular characterization should be mandatory for all vaccines used against LSDV prior to use*(Eeva et al;2016).*In performing this experiment, our objective was to trial strategies for DNA extraction, sequencing and analysis of LSD virus harvest which may considered as a one-step quality control for vaccine production as homologues vaccine preparation and a nanopore sequencing method to detect and characterize tissue culture propagated LSDV as well as classification of microbial taxa un biased manner. Attenuated LSDV was obtained and propagation is done for virus activation on confluent MDBK cell cultures ,CPE increased daily till reaching maximum by day 6(90%)as showed by(*Aboul –soud ;1996)* the activated virus was titrated on MDBK cell the titer was $10^{5.5}$ $TCID/_{50}ml$ then the titrated virus was taken for DNA extraction using phenol chloroform extraction and concentration as the minimum input of DNA should be ~400ng input mass measured by qubit *(oxford NANOPORE technologies*) for nanopore sequencing . The library preparation protocol for the sample includes the ligation of adapters via a T4 ligase. The data is operated with the MinKNOW software program and saved as a raw data(FAST5 file) this data is collected and converted to nucleotide sequence Using Albacore software(FASTQ) data the sequencing data are shown in different taxonomic resolutions, and a detailed data especially in species level . At the species level, nanopore sequencing data had better resolution than the short-read sequencing data *(Viehweger et al;2018)* all this data is achieved because of the technology's ability to detect all what in the sample as shown in fig.(7).This data in this number of reads may indicate the presence of this organisms but in a non-replicative form due to treatment of all ingredients used

for virus propagation in order to decontaminate and sterilize them prior to use in the vaccine preparation. In addition, some others may be due to presence of high error rates especially in regions of low coverage or where the underlying reference assembly is erroneous this high sequence error rate remains a challenge (*Quick et al., 2015, 2016; Hoenen et al; 2016*) to improve the technology ,this is because the re- squiggle algorithm – upon which this method is based to align the raw nanopore read signal to the base called read sequence(*Marcus et al;2016*) ,it may also be related to reagent stability and the integrity of individual pores on the flow cell(*Benjamin L. et al; 2019).* Although this technique is extremely fast producing more than 8K in less than 7.5 hours as shown in fig(6) ,but this speed decrease the sequence resolution because the DNA strand pass rapidly at the rate of 1 to 5μs per base through the nanopore (*Daniel. F. et al; 2008*).

Therefore our study may need further conventional studies in parallel with as virus isolation, real time PCR and plaque purification for more confirmation due to a lack of sensitivity and multiplexing in such method (*Keller, M. W. et al; 2018*)

The number of lumpy skin reads was 1,417 taking the majority of reads whereas goat pox 233 and finally sheep pox virus 187 cumulative read as shown in fig (4). This result is probably due to high Genetic similarity between them with more than 95% identity (*Tulman et al;2001*).

The number of reads identity for GPV is shown to be more than that for SPV which indicate the higher similarity between LSDV and GPV than to SPV as discussed recently by informing that the Kenyan vaccine virus was actually a GTPV strain (*Omoga et al; 2016),* In addition Kenyan sheep and goat pox (KSGP) virus O-240 and 180 vaccines were identified to be actually LSDV virus (*Lamien et al; 2010*).

CONCLUSION

In conclusion this tool is useful to qualify the overall virus seed as an input sample to standardize and characterize a seed in vaccine production together with real time data which is an indicator for virus amount and purity of a master seed virus, so it could be considered as a comprehensive tool for in process quality control of LSDV harvest.

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