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RESEARCH

Investigation of the replication capacity of lumpy skin disease virus in ticks

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ABSTRACT

Background: Lumpy skin disease (LSD) is a highly contagious trans- boundary disease of cattle with major economic losses. Lumpy skin disease virus (LSDV) belongs to the genus *capripoxvirus* of *Poxviridae*. Blood-feeding arthropods like mosquitoes, flies and, African tick species play a role in LSDV maintenance and transmission. Mechanical transmission of LSDV is well documented; however, evidence of transovarian and transstadial transmission is a tick warrants an investigation into the replication capacity of the virus in ticks. Transstadial occurs when virus remains with vector from one life stage to the next and transovarian is transmission of virus from parent vector to its egg.
Objective: Investigation of LSDV replication capacity in ticks uses targeted detection of the viral DNA polymerase mRNA and metagenomics analysis using MinIon technology.
Methods: A total of 34 adult female ticks were collected from three LSD clinically disease cattle that were previously vaccinated using the Egyptian sheep pox (SPPV) vaccine, apparently healthy cattle in contact with the diseased cattle, and one healthy animal located in a previously infected zone. LSDV-specific PCR was used to detect LSDV in sampled ticks. PCR-positive tick samples were subject to a modified RT-PCR assay to detect the polyadenylated LSDV DNA polymerase mRNA. Metagenomics analysis using MinIon technology was used to generate LSDV sequences and to investigate relative abundance of LSDV sequences in the sampled tick population compared to other known organisms detected.
Results: Five ticks randomly selected from those collected were PCR positive for LSDV. 6 ticks randomly selected from the collected ticks were pooled and this pool was also positive for LSDV using PCR. The modified RT PCR gave inconclusive results. Metagenomics analysis indicated the presence of a high genomic load of different known commensal and pathogenic microorganisms that have been reported to replicate in ticks (e.g. *Staphylococci, streptococci*) In addition, the analysis revealed a LSDV sequence output that was nearly equal to sequence output of different bacteria known to replicate in ticks.
Conclusion: The modified RT-PCR assay used to detect the polyadenylated LSDV DNA polymerase mRNA in combination with data generated using metagenomics analysis provided additional support to the hypothesis that LSDV can replicate in ticks and, warrants further investigation into the replication capacity of LSDV in ticks.

Keywords: LSDV; Metagenomics analysis; DNA polymerase; RT PCR, MinIon technology.

BACKGROUND

Lumpy skin disease (LSD) is an infectious viral disease of cattle that is caused by lumpy skin disease virus. (LSDV) is a member of *Capripoxvirus* of *Chordpoxvirinae* of *Poxviridae*. Despite causing low morbidity and mortality, LSD has great economic significance due to extensive damage of hides, drop in milk production, infertility, restriction of animal movement and cost of control and eradication measures (*Green 1959; Weiss 1968; Tuppurainen et al. 2012*), LSD is associated with significant production losses. It is therefore defined as a notifiable disease by the *World Organization for Animal Health (OIE*).Blood-feeding arthropods e.g.

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(Aedes aegypti and Rhipicephalus appendiculatus)play role in LSDV maintenance and transmission either mechanically or biologically (*Lubinga et al. 2013a; Lubinga et al. 2014c and Tuppurainen et al. 2011*).In 2013, Tuppurainen reported the passage of LSDV from infected female ticks through the eggs to the next generation larvae of A. hebraeum, R. appendiculatus and R. decoloratus, as well as transmission to recipient animals by A. hebraeum and R. appendiculatus larvae (i.e.Transovarial transmission) (*Tuppurainen et al. 2013b*).A.hebraeum can survive without blood meal for long time (several years); thus contributing to virus epidemiology if the virus survives or replicate in them (*Sonenshine 1991; Walker2003*).

To investigate whether a virus replicates in an arthropod vector or not, it is imperative to detect increase in virus titer (which is difficult) or, detect virus gene expression; for example, detect the expression of genes involved in replication or immune evasion. However, since LSDV is a DNA virus, direct detection of ORF segments would have to be preceded by a DNase treatment step or an alternative approach to avoid detection of genomic viral DNA. Alternatively, robust metagenomics analysis can be used to investigate the relative abundance of viral nucleic acid in relation to other microorganisms that normally inhabit, or replicate in, ticks. Such an analysis would provide data that can be used to support the possibility of virus replication in a host; especially when combined with other diagnostic approaches.

Oxford Nanopore sequencing technology is a next generation technology that has been shown to generate massive amounts of genomic data from low quantities of DNA from environment. The use of Minlon (Oxford nanopore technologies, UK) guarantees generating relatively unbiased data especially in the absence of prior knowledge of the sample content (*Jain M, et al. 2005; Brown C, 2012*).

MATERIALS AND METHODS

Animal selection, sample collection and preparation

A total of 34 adult female ticks were collected from three LSD clinically disease cattle that were previously vaccinated using the Egyptian sheep pox (SPPV) vaccine (sheep poxcapri-vac, veterinary serum and vaccine research institute vaccine).), apparently healthy cattle in contact with the diseased cattle and, one healthy animal located in a previously infected zone. Ticks were collected from different parts of animal's body; including ears, neck, shoulders and, back. Ticks were transported on ice to the laboratory, and then kept frozen at -25 °C until tested.

DNA and RNA extraction:

The cuticle of ticks was removed and the viscera with hemolymph of ticks were recovered; Extraction was done using (Qiagen DNeasy® Blood & Tissue kit) according to (*Ard M Nijohf et al.2009*). However, DNA/RNA extracts from control spiked tick viscera were negative for LSDV DNA. Therefore, a modification was developed to enhance recovery. The technique included the following modification: [1] manual homogenization of sample [2] duplication the amount of tissue lysis buffer (ATL) adding with it RNA carrier to keep integrity of RNA [3] duplication the amount of cell lysis buffer (AL) adding with it RNA carrier [4] duplication the amount of proteinase k. Confirmation of DNA recovery from ticks using the modified approach was done by detection of a tick house-keeping gene which is elongation factor 1(Table 1); (ELF1A) was chosen as an internal control due to it is the most stable expressed gene in comparison to other housekeeping genes in ticks (*Ard M Nijohf et al.2009, Esteves E., et al, 2007).*

PCR amplification:

A conventional PCR was used to amplify a 179bp region within the capripoxvirus ORF 103 that encodes the DNA polymerase gene (pan capripoxvirus primers; table 1) (Balamurugan et al. 2009). MyTaq Red PCR Master Mix (2X) (*bioline,USA*) was used. Five microliters of each extract, positive DNA extracts or, nuclease free water as no template controls were used. Thirty-five PCR amplification cycles were done in a thermal cycler (Applied Bio systems™). Under the following conditions: initial denaturation at 95℃ for15 s, annealing at 62℃ for 15 s, and extension at 72℃ for 10 s. A final extension at 72℃ for 10min was done following cycling. Five micro liters of amplified amplicons were separated on 1.5% ethidum bromide stained agrose gels at100V for 30 min. The amplified amplicons were visualized in comparison with 1 kb plus DNA ladder (Thermo fisher scientific, US) using a transilluminator.

RT PCR was expected to amplify the 1345 bp region within the expected m RNA of *capripoxvirus* by using of reverse primer of pan capripoxvirus primer and poly T primer, the quantity of poly T was 8x to normal to increase specifications as, ticks had housekeeping genes plus other microorganisms genes so competitions on poly T primer would be high (table 1). One-step RT-PCR premix kit (*Intron biotechnology, South Korea*) was used. Five microliters of each extract, positive DNA extracts or, nuclease free water as no template controls were used. One cycle for RT enzyme was under the following conditions: 50℃ for 5 min, 42℃ for 60 min, 95℃ for 5 min, then forty PCR amplification cycles were under the following conditions denaturation at 95℃ for 45 s, annealing at 60℃for 30 s, and extension at 72℃ for 3 min. A final extension at 72℃ for 10min was done, all these cycles was done in a thermal cycler (Applied Bio systems™) following cycling. Five micro liters of amplified amplicons were separated on 1.5% ethidum bromide stained agrose gels at100V for 30 min. The amplified amplicons were visualized in comparison with 1 kb plus DNA ladder (Thermo fisher scientific, US) using a transilluminator.

Table (I): Primers

primer	sequence	Location
Pan Capri.Forward primer	5’GGAATGATGCCRTCTARATTCCTATC 3’	32,922–32,947*
Pan Capri.Reverse primer	5’CCCTGAAACATTAGTATCTGTATTTGTTGC 3	33,072–33,101*
Poly T	5’TTTTTTTTTTTTTTTTT3’	
ELF1A -F	5’CGTCTACAAGATTGGTGGCATT3’	39-60**
ELF1A -R	5’CTCAGTGGTCAGGTTGGCAG3’	147-128**

* Reference sequence used Gene Bank#: AY077836 for pan capripoxvirus primer
**Reference sequence used Gene Bank#:EW679365 for elongation factor 1alph

MinION nanopore sequencing

Sample preparation was done for positive LSDV sample, DNA was quantified using Qubet fluorometer 2.0, were used. Rapid bar-coding sequencing kit (SQK-RBK004) depending on template molecules were cleaved by transposes, then barcoded tags attached to cleaved ends, after that barcoded sample pooled then Rapid Sequencing adaptors are then added to the tagged ends, library preparation was done through using the ONT 1D Genomic DNA ligation (SQK-RBK004) protocol. Then sequencing was done through flow cell then fast5 data files were base-called using the ONT Albacore module

RESULTS

PCR findings

Conventional PCR: Five randomly ticks were positive and LSDV using pan caprivirus primers (Fig. 1, 2), one pool (6 ticks) was positive LSDV using pan capripoxvirus primers (Fig. 3), treated samples with DNaesy enzyme which were still LSDV DNA positive (Fig. 4), [RT PCR]: modified RT PCR assay was done for positive LDSV pool, which giving a band at 1345 bp (Fig.5)

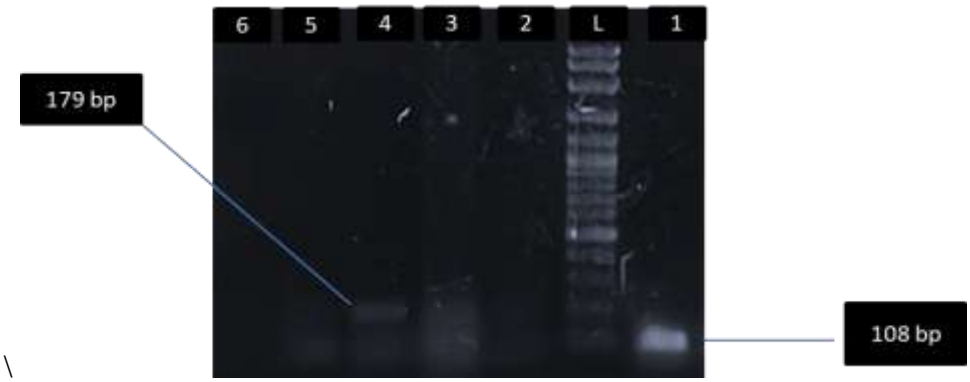

Fig. 1. Detection of LSDV DNA in tick samples using pan capripoxvirus primer: L: DNA ladder 100bp plus, lane1: Internal positive control ELF1A, lane (2, 3, and 5) negative LSDV DNA samples, lane 4: positive LSDV DNA sample. Successful DNA extraction from ticks was confirmed using ELF1A tick primers

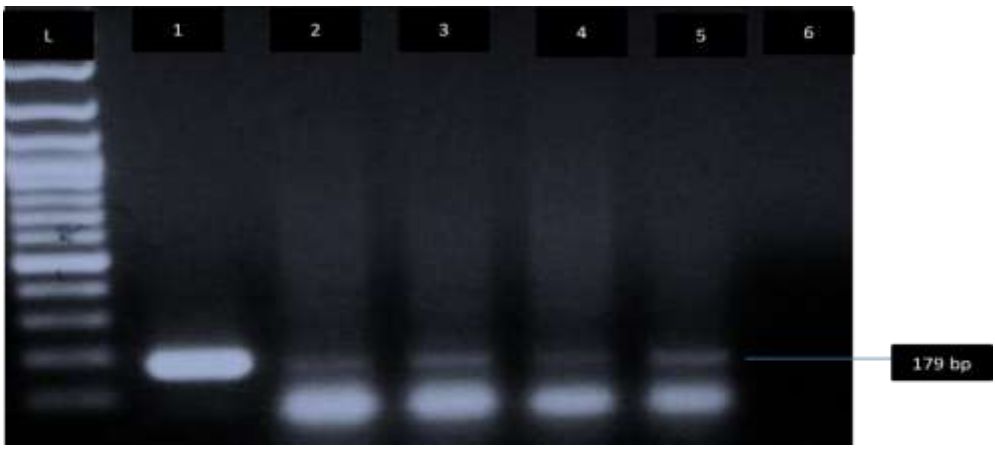

Fig. 2. Detection of LSDV DNA: L: DNA ladder 100bp plus, lane1: positive control for testing reaction (sheep pox vaccine), lane (2 – 5) positive LSDV DNA samples, lane 6: no template control

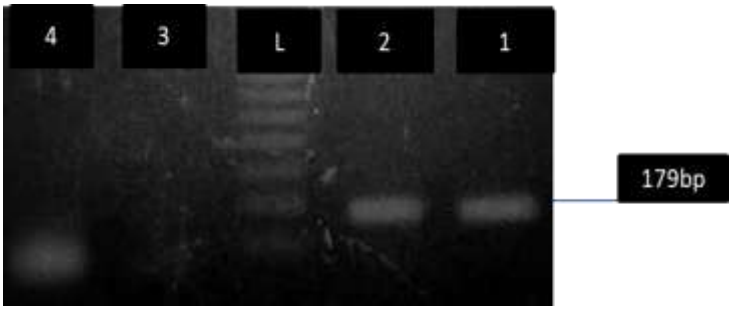

Fig 3. Detection of LSDV in random pools of six ticks using the pan capripoxvirus: lane 1: Positive control (sheep pox vaccine), lane 2: Positive LSDV DNA pool, L: DNA ladder 100bp plus, lane 3: no template control, lane 4: negative LSDV DNA pool;

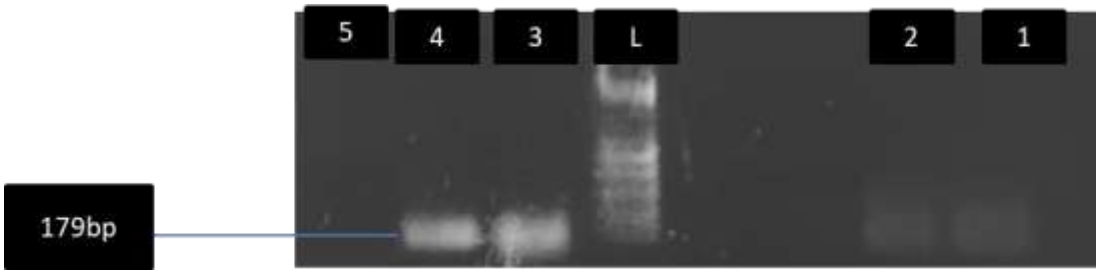

Fig.4: Confirmation of the efficacy of DNAse treatment by detection of LSDV DNA in treated sample using pan capripox primers': DNA ladder 100bp plus, lane (1, 2) sheep pox vaccine used as positive control for testing DNaesy efficacy, lane (3, 4): DNase-treated positive LSDV DNA samples. Lane (5): no template control. Results indicate that DNase treatment did not completely remove viral DNA from DNA/RNA extracts.

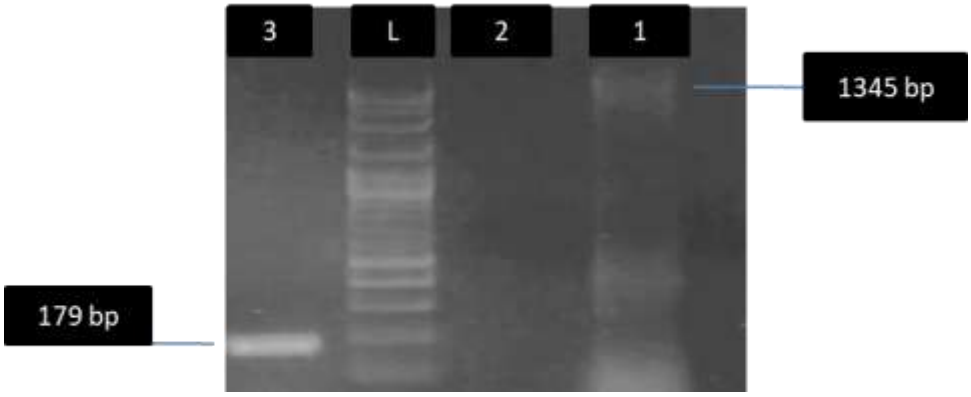

Fig.5: Amplification of viral DNA polymerase gene expression using the modified RT-PCR assay. L: DNA ladder 100bp plus; lane 1: expected positive LSDV RNA pool; lane 2: no template control; lane 3: Master Mix control (DNA extract from sheep poxcapri-vac, veterinary serum and vaccine research institute vaccine). The expected mRNA length is 1345 bp

Metagenomics analysis results

The Nano pore sequencing run workflow was 99.9% successful; generated 124525 successful reads. Only 110 failed reads were reported. The yield per hour was 30 million bases (Fig.6). The quality score showed a higher confidence in the classification of the taxa. for the

input DNA sample (Fig.7)and, allowed interpretation of results down to the genus and species levels(Fig.8).Sample composition, which is a donut chart showing the relative proportions of reads from the species found in the sample, indicated a significant presence of viral genomes (Fig. 9). Metrichor WIMP application report showing numerous numbers of different bacteria, fungi and viruses (Fig. 10)

Fig. 6: The work flow succeeded in almost all barcodes included in the run. Only barcode 4 and 5 were pertinent to this experiment.

Fig. 7: quality score.

Fig. 8: Nanopore sequencing results allowed taxa classification at genus level.

Fig. 9: The circle shows the percentage of reads for each super kingdom.

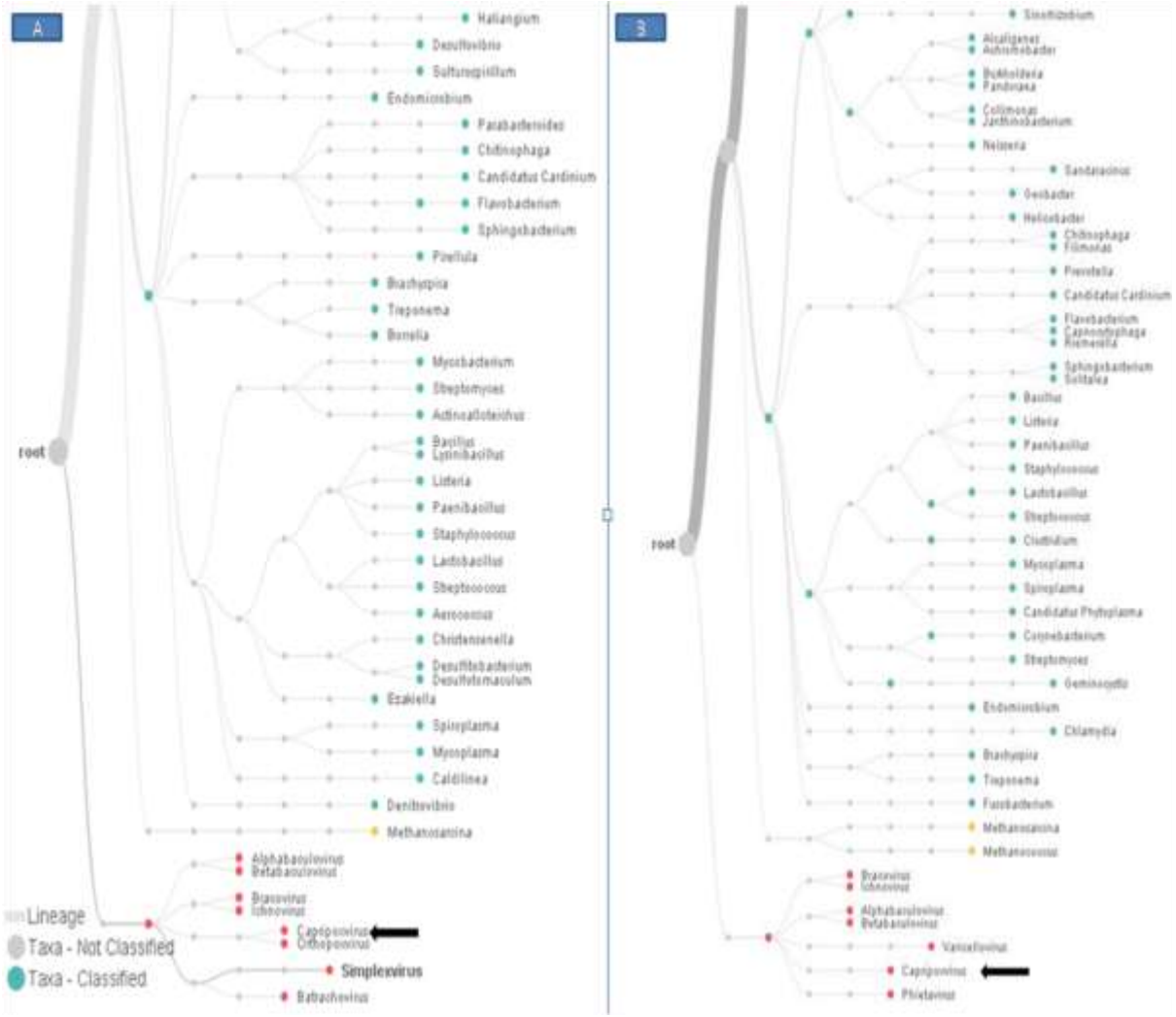

Fig. 10: Metrichor WIMP application report for some known commensal and pathogenic microorganisms that have been reported to replicate in different tick samples collected at different times and places. The subtree representing the NCBI taxonomy and thickness of the branches is proportional to the count reads along the lineage. [A] Tick sample collected from Benha, from two years ago [B]. Tick sample collected from Touk, from one year ago

Table (II): Number of hits in metagenomic datasets matching selected species containing pathogenic strains in tick sample (per 10 micrograms)

Species	Number of hits
Phietavirus (bacteriophage)	3
Streptococcus mutans	2
Lactobacillus acidophillus	4
Staphylococcus aureus	4
lumpy skin diseases virus	3
Listeria monocytogene	8
Mycobacterium leprae	3
Bacillus anthracis	5

DISCUSSION

Lumpy skin disease is a highly contagious disease causing massive economic losses. LSDV persistence in the environment between outbreaks is multifaceted. A thorough understanding of the viral mechanism of survival in the environment is needed for success of control and eradication efforts in endemic countries. The role of ticks as a possible biological vector for LSDV has been investigated using several approaches. In 2014 Lubinga reported that LSDV was detected in engorged and non-engorged ticks by using monoclonal antibodies with immunohistochemical staining, and also the viral antigen was detected in salivary glands, hemocytes, synganglia, ovaries, testes, fat bodies, and concluded that detection of viral antigens in these organs suggested that these organs could be the survival sites for LSDV during transstidial passage and crossing of LSDV mid gut wall was strong evidence about biological transmission of LSDV (*Lubinga, et al 2014*). Although this is strong supportive evidence for replication, it did not unequivocally demonstrate virus replication as there could be mechanisms for persistence of virions without replication in such tissues.

In 2013.Tuppurainen reported that transstadial and transovarial passage of LSDV from infected A. hebraeum, R. appendiculatus, and R. decoloratus female ticks to larvae of the next generation through their eggs was demonstrated. The detection of LSDV in saliva of orally infected adult A.hebraeum and R. appendiculatus ticks fed as nymphs or as adults on experimentally infected donor animals, suggests the potentiality of the virus to reach the salivary glands before repletion and hence can be transmitted to another host following interruption of feeding (*Tuppurainen, et al 2013*). However, this too did not unequivocally demonstrate replication because there was a suspicion that intact virions may be maintained in tissues that are not lost during tick development. Also, it is very difficult to assess viable virus titer in the different stages of the tick life cycle.

The approach adopted here initially depended on attempting to unequivocally demonstrating viral DNA polymerase mRNA expression in tick

tissues; viral DNA polymerase is only needed during cytoplasmic replication. Primers flanking conserved region of the target gene would amplify viral DNA even in a RT-PCR reaction. Hence, DNase treatment was attempted to remove viral DNA from mixed DNA/RNA extracts prior to the RT-PCR step. Since complete removal of viral DNA was not possible, modified primers were designed to target the poly (A) tail of the mRNA; the poly(A) tail is added later in mRNA morphogenesis. The mechanism of mRNA poly(A) addition involves J3-homologue enzyme activity (REF). The J3 poly (A) polymerase catalyzes addition of 35 A residues on the 3` end of m RNA, then after tail grows to 35 bases, poly (A) polymerase ceases rapid elongation of the A tail and slowly add A residues at rate 0.25- 1 per minute, then poly (A) polymerase returns to processive mode of elongation till it reaches ~ 100 nt (*Gershon, 1998*).

An RT-PCR of the expected size was generated using the modified RT-PCR assay. However, additional PCR products were visible in the visualized product. This was expected since mRNAs and poly (A) tails may vary in size during morphogenesis (*Gershon, 1998; Lanter et al. 2002*). Attempts to sequence the PCR product generated low-quality reads. Therefore, a shift towards a more robust approach for gleaning data regarding relative virus genome abundance in tick gut extracts was adopted.

Metagenomics analysis was used as supportive tool for investigating the possibility of LSDV replication in ticks. Metagenomics analysis involves the application of bioinformatics tools to study the genetic material from environmental microorganisms and classify sequences to known functions or taxonomic units based on homology searches against a database (*SisselJuul. et, 2015*). Thus, theoretically, metagenomics can detect all DNA in a given sample (virus and eukaryotes) without bias *(SisselJuul. et, 2015*).

In metagenomics analysis, organisms with larger genome sizes generate more reads even though the organism may not be more abundant in samples (*Etienne Yergeau,et. 2016)*. This means that the majority of reads will be for the tick DNA, followed by DNA from bacteria. On the other hand, DNA of bacteriophages should also be present in considerable amounts since its replication in bacteria means that it will be present in numbers several logs higher than those of bacteria. This picture was indeed present in the data generated (Fig. 9).

Relative abundance of DNA in metagenomics analysis data was used as confirmatory tool for detection of relative abundance of different microorganisms in sewage water before and after treatment (*Etienne Yergeau,et. 2016*). Data generated using metagenomic sequencing was consistent with, and sometimes more sensitive than, that generated from quantitative real-time PCR and/or cultivation. Thus, changes in microbial titers following treatment was detected for pathogens such as *Enterococcus, Campylobacter,Staphylococcus,Yersinia and*

*Closterdium*spp., even when cultivation was sometimes not possible or impractical *(Etienne Yergeau,et. 2016).*

In our study sequence output/load (number of sequences identified not number of pathogens) of *Capripoxvirus* could be compared to sequence output/load of other known commensal and pathogenic microorganisms that have been reported to replicate in ticks (*Streptococcus mutans*, *Listeria monocytogene*, *Staphylococcus aureus*,table ii). Sequence output for LSDV was nearly equal to that of (*Streptococcus mutans*,*Staphylococcus aureus* and *Phietavirus*). These organisms are known to replicate even on inanimate organic material and have much larger genomes compared to the virus, thus, the data presented here suggest an abundance of the LSDV genome indicative of replication not just mechanical transfer. This is despite the fact that the relative number of reads for key sequences (like some bacterial genomes and phage sequences, table ii) was much less than that of the study referenced above (*Etienne Yergeau,et. 2016*); only 10 mg of tick viscera and hemolymph were sampled in our study compared to 10 gm sampled in the biosolid study (*Etienne Yergeau,et. 2016*).

Taken together, the modified RT-PCR assay used to detect the polyadenylated LSDV DNA polymerase mRNA in combination with data generated using metagenomic analysis provided additional support to the hypothesis that LSDV can replicate in ticks and, warrants further investigation into the replication capacity of LSDV in ticks using cell culture technology and transcriptome analysis.

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