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## In silico design of short hairpin RNA (shRNA)Molecules for DNA pol gene of Contagious Ecthyma virus (ORFV)

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**ABSTRACT:** Contagious ecthyma is an infectious skin disease of ruminants caused by the ORF virus (ORFV) that is a member of genus Parapoxvirus of the Poxviridae family. In addition to the significant effects on lambs and human, ORFVs have been recently shown to infect other hosts. The disease causes significant economic damages to the sheep industry, so attempts to eliminate it must be taken into account. RNA interference (RNAi) is an evolutionarily conserved mechanism in which the expression of homologous target genes is suppressed by means of double-stranded RNA molecules. Since RNAi can be considered as a therapeutic method for viral gene silencing, we tend to make the most of this capability. The present study aims to design potential shRNAs to knockdown the DNA-polymerase gene coded by ORF025. A significant number of computational methods such as clustal omega website to target alignment, BLAST-NCBI to similarity search, CLC software to secondary structure prediction, BLOCK-iTRNAi Designer and WI siRNA Selection Program and Software to design of shRNA molecules and scoring have been applied for shRNA molecules designing against the ORF025-DNA pol gene of ORFV. Then three shRNA molecules were logically designed against the ORF025-DNA pol gene. In conclusion the present study provides a strong and superior approach for achieving a validated strategy to design an antiviral shRNA molecule that meets many sequence features for efficient ORFV knockdown and treatment at the mRNA level. The efficiency of these anti ORFV shRNAs need to be tested

**Keywords:** In silico, knockdown, ORFV, RNAi, shRNA

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## INTRODUCTION

Contagious ecthyma or orf is a life-threatening skin disease affecting ruminants worldwide (Nandi, De, & Chowdhury, 2011; Zeedan, Abdalhamed, Ghoneim, & Ghazy, 2015). The causative agent of the disease is the *ORF virus (ORFV)* which is a member of the genus *Parapoxvirus* of the family *Poxviridae*. The disease is significant, especially in young lambs. The most common sites for those orf signs occurring are around the mouth, teats and, skin surface. The lesions are proliferative and self-limiting. Notably, that contagious ecthyma is a risk to the sheep industry and threatens human health (Wang et al., 2014)

The genome of ORFV is a linear double-stranded DNA, 134-139-kb (Zeedan et al., 2015). Since ORFV can be replicated within the host cell cytoplasm, it needs to encode its DNA transcription and replication (Fleming, Wise, & Mercer, 2015). One of the most essential proteins in the replication of ORFV is the DNA polymerase (DNA Pol) encoded by the virus and plays a key role during ORFV replication (Wang et al., 2014). In contrast, vast efforts to discover the ORFV pathogenesis, none of the available vaccines induces complete and long-term immunity and vaccinated animals may be re-infected (Bergqvist, Kurban, & Abbas, 2017).

The potential of RNAi for efficient and specific target gene silencing has increased efforts to develop several therapeutic agents based on RNAi (Barata, Sood, & Hong, 2016). RNAi can be induced by the introduction of synthetic small interfering RNAs (siRNA) or by the intracellular generation of siRNA through vector-driven expression of the small precursor hairpin (sh)RNAs (Manjunath, Wu, Subramanya, & Shankar, 2009). There are also evidence that shRNAs may be more effective in gene silencing than siRNAs and induce lower non-specific gene expression changes (Lambeth & Smith, 2013). It is noteworthy that, the most benefit shRNA than siRNA is that it is capable of cloning in to a viral vector and enter into numerous cell lines (Haussecker, 2014).

Given the high efficiency of RNAi to silence target genes and the crucial role of DNA-pol to replicate ORFV genome, the present study aimed to design potential shRNA molecules against the ORF025-DNA-pol gene of ORFV.

## MATERIALS AND METHODS

### Selection of ORF025-DNA pol conserved regions

The complete sequences of ORF025 (ORFV-OV-

SA00 strain) were obtained using the NCBI GenBank database (accession number AY386264.1). In order to identify the conserved regions among ORF025-DNA pol gene, the complete CDS of ORF025-DNA pol was aligned with other similar strains using clustal omega website, The ORFV-OV-SA00 strain was used for shRNA designing.

The noticeable thing is that one mismatch between the target mRNA and shRNA has a profound effect on shRNA efficiency. Using this rule ensures that all the designed shRNA molecules belong to the consensus regions of transcripts. Therefore, the regions of transcripts with maximum homology are selected for the following analysis.

### Design of shRNA molecules against ORF025-DNA pol gene

The design of shRNA molecules was performed by three online websites as performed by three online websites: BLOCK-iTRNAi Designer, WI siRNA Selection Program, and Software. These websites have their advantages. All shRNAs offered by these websites were aligned with the completed CDS of ORF025-DNA pol to detect more appropriate shRNAs. After designing shRNA, both sequence and structural rules must be taken into account; however, according to the evidence, the most significant determinant of the potency of the silencing trigger is the sequences of shRNAs (Paddison, Caudy, Bernstein, Hannon, & Conklin, 2002). Briefly, several sequence rules are as follows: The target region should not be placed in 50-100 nucleotides of the start codon and the termination codon as well as intron regions or single nucleotide polymorphism (SNP) sites; The target sequence needs to have a GC content <30% or > 60% providing the vital stability for the shRNA (Protocol-online, 2009). McIntyre et al. found it difficult to find a fixed correlation between stem length and suppressive activity, and described that core placement at the base terminus is essential for appropriate activity (McIntyre et al., 2011). A significant number of sequence rules denoting the preference or avoidance of the specific positions belonging to both the sense or antisense strand of the duplex have been reported (Protocol-online, 2009). Structural rules characterize thermodynamic properties in terms of the secondary structures and accessibilities of shRNA/target site (Baghban-Kohnehourz & Nayeri, 2016).

**Table 1:** Characteristics of Effective shRNA Molecules Targeting ORF025-DNA pol gene

Target No.	Location of Target Within mRNA	Sense strand sequence	Length	GC, %	1*conserved DNA sequence	2*unconserved DNA sequence	U at position 10 (sense)	proper regions of the RNA secondary structure	crowded regions of the RNA secondary structure
1(OrfshRNA-1)	959-980	GCACCACCTTCCACATCAACA	21	52.39	+		+	+	
2	963-985	CACCTTCCACATCAACAACAACA	23	42	+		+	+	
3	959-978	GCACCACCTTCCACATCAA	19	52.63	+		+	+	
4	960-982	CACCACCTTCCACATCAACAACA	23	47	+			+	
5(OrfshRNA-2)	894-916	GACAGTCAACTTCTGCGTGTACG	23	47	+		+	+	
6	14-36	TGGAGCTGAAATGTTTGAAGTGG	23	37	+		+	+	
7	17-39	AGCTGAAATGTTTGAAGTGGTTC	23	37		-	+	+	
8	2706-2728	AACGCACCACAAGAAGTCAAGT	23	47	+			+	
9	2708-2730	CGCACCACAAGAAGTCAAGTCC	23	42	+			+	
10	2705-2723	GAACGCACCACAAGAAGT	18	47.37		-		+	
11(OrfshRNA-3)	1649-1671	ACGTGCTCATCTTCGACTACAAC	23	47		-	+	+	
12	1651-1670	GTGCTCATCTTCGACTACA	19	47.37		-	+	+	

\*1: Linked to the entirely conserved DNA sequence

\*2: Linked to unconcerned DNA sequence

On top of that using the shRNA design tool, the shRNAs-suggested were manually investigated according to the parameters proposed by Tom Tuschl's rules (Protocol-online, 2009), McIntyre et al. (McIntyre et al., 2011), Bofill-De Ros et al. (Bofill-De Ros & Gu, 2016) and Fakhr et al. (Fakhr, Zare, & Teimoori-Toolabi, 2016) for optimal designing.

#### Alignment of shRNA with sheep genomic

In order to eliminate shRNAs having off-target effects on non-targeted genomes, a BLAST homology search with sheep genomic and transcripts database (<http://blast.ncbi.nlm.nih.gov/>) was performed. shRNAs having a perfect match of 16 nucleotides or more with any other mRNAs of the same species were excluded from the suggested list of shRNAs (Taxman, Moore, Guthrie, & Huang, 2010).

#### Secondary structure prediction of ORF025-DNA pol

The ORF025- DNA pol secondary structures were predicted using CLC software (CLC Genomics Workbench). In order to detect that the shRNAs are matched with the appropriate regions of the structure, they were blasted with this predictable secondary structure. The shRNAs that were placed in stem and crowded regions were excluded from consideration.

#### Choosing shRNAs with the best score

Afterwards, the shRNAs which remained characteristics such as sequence specificity, off-target effects, and G/C content, were investigated once again. Finally, three shRNAs with the highest scores were

selected. Nucleotide sequences TCAAGAG, as a loop sequence, were added between the sense and anti-sense strands.

#### RESULTS

Around 11 DNA pol gene sequences from various strains of ORFV were obtained from the data base nucleotide sequence, NCBI. The clustal omega was used for detecting the consensus region by multiple sequence alignment. The shRNAs were designed based on the conserved regions of ORF025. BLOCK-iTRNAi Designer, WI siRNA Selection Program and wizard websites were used for shRNA designing. WI siRNA Selection Program was also able to show off-target effects on the seed complementarity of the target site among the related species.

Some shRNAs were obtained by BLOCK-iTRNAi Designer, the WI siRNA Selection Program and wizard server, respectively. Then they were manually and accurately investigated in order to design the most potent molecules. Using such scoring system, an acceptable score remarkably raises the eventuality for gene knockdown. Finally, the number of shRNAs were determined to stick to the scoring system rules (Table 1). Before the final scoring, all shRNAs were analyzed to demonstrate the specificity of shRNA by BLASTN. Since all the shRNAs were proprietary, this step has been deleted from the final scoring. All shRNAs retain GC content within 37% - 52%. Finally, three of the shRNAs were selected with the highest scores (Table 2).

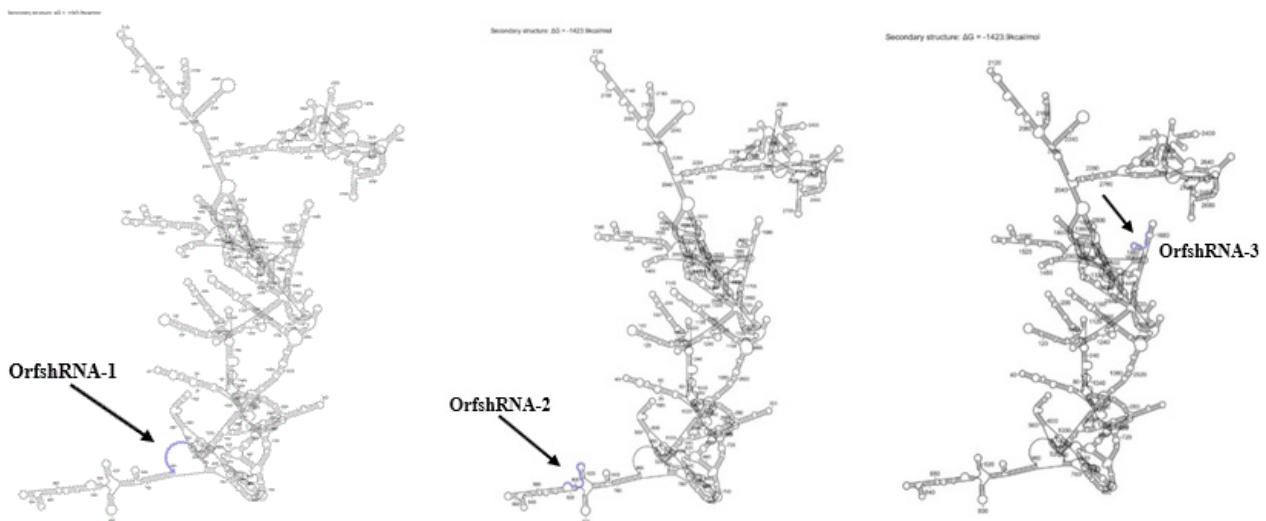
The CLC software was used for predicting the

mRNA secondary structure and the interaction between shRNAs and mRNA, as shown in figure.1. Fi-

nally, shRNAs namely OrfshRNA1, 2, 3 have more accessibility for target recognition (Table 2)

**Table 2:** Characteristics shRNAs with the best score

Target Name	Sense Sequence	loop sequence	Antisense sequence
OrfshRNA-1	GCACCACCTTCCACATCAACATCAAGAGTGTGATGTGGAAGGTGGTGC		
OrfshRNA-2	GACAGTCAACTTCTGCGTGTACGTCAAGAGCGTACACGCAGAAGTTGACTGTC		
OrfshRNA-3	ACGTGCTCATCTTCGACTACAACCTCAAGAGGTTGTAGTCGAAGATGAGCACGT		



**Figure 1:** The arrows indicate the location of interaction shRNA with the mRNA

\*shRNA Target Sites (black) on the ORF025 mRNA Secondary Structure Predicted Using CLC

## DISCUSSION

Since the available vaccines are not able to provide complete protection, ORFV is capable of reinfection the same individual and that is what makes disease control very tough (Hosamani, Scagliarini, Bhanuprakash, McInnes, & Singh, 2009).

As time has passed, gene therapies through RNAi, particularly to treat viral diseases, have become more and more attractive and promising (Qureshi, Tantray, Kirmani, & Ahangar, 2018). We can benefit from shRNA technologies due to permanent integration of expression constructs for long-term expression, the use of viral vectors to infection of cell lines and tissues that is hard to target, and the temporal control of shRNA transcription by means of inducible promoters (Lambeth & Smith, 2013).

The central region of the viral genome includes the DNA pol gene that codes for a highly conserved non-structural protein playing an initial role in viral proliferation. This gene is exceptionally conserved across the species belonging to *Parapoxvirus* (Bora et al., 2011; Li et al., 2013). It can be stated that knock-down of the DNA pol gene will disturb its critical functions. The researchers have shown that the lower GC content is more effective for hybridizing the two different RNAs (Kanasty, Whitehead, Vegas, & Anderson, 2012).

The lack of effective drugs against most viruses and the definitive treatment of viral diseases are undeniable facts. The treatments are merely supportive. For this reason, the therapeutic application of RNAi is of interest to virologists since it provides exact and swift targeting of viral genes (J Blake, F Bokhari,

& AJ McMillan, 2012). Yin et al. (2010) have used shRNAs to inhibit NDV replication by targeting the viral matrix protein gene (Yin et al., 2010). Zhao et al. (2012) have carried out another study targeting the ORF095 gene of the Goatpox virus by shRNAs design (Zhao et al., 2012). Wang et al. (2014) applied siRNAs against the DNA pol gene of ORFV and displayed 73-89 % less than viral DNA in transfected cells (Wang et al., 2014). However, due to the pros of shRNA molecules over siRNA above-mentioned, in the present study, shRNA molecules were applied to prevent ORFV replication.

In this study, shRNA molecules were designed by means of BLOCK-iTRNAi Designer, WI siRNA Selection Program and wizard websites as above-mentioned. The mRNA secondary structure and the interaction between shRNAs and mRNA were predicted. Afterwards, to design the most potent molecules, we manually and accurately checked some options thanks to a proper scoring system: such as GC %, conserved DNA sequence, U at position 10 (sense), analyzing the specificity of shRNA by BLASTN, proper regions or uncrowded regions of the RNA secondary structure, etc. as above-mentioned. To sum it up, three of these molecules were elected with the highest scores. Experimental studies suggest that constructs with a pyrimidine rich loop sequence and short hairpin stems (19 nucleotides) are profoundly influential for prosperous shRNA design. The short hairpin (18-21 nucleotides); pyrimidines do not pair, and the use of uridines in the loops are the best conditions

for designing effective shRNA molecules (Matveeva et al., 2010). The presence of internal repeats or palindromes in siRNA constructs may lead to internal fold-back structure formation consequently may reduce knockdown efficiency (Reynolds et al., 2004). Finally, these rules provided us with the chance to design powerful and strong shRNAs molecules as best as possible.

In the future, we tend to apply a lentiviral vector to gain a constant expression of the designed shRNAs to prevent ORFV replication for a long time.

## CONCLUSION

Concerning the overall outcome of the observations, the application of new strategies for the treatment of orf disease is imperative. Due to the numerous pros of shRNAs, using these molecules provides researchers with the chance to control this contagious infection. Also, this method seems absorbing and can slow down or even stop the progression of the disease.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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