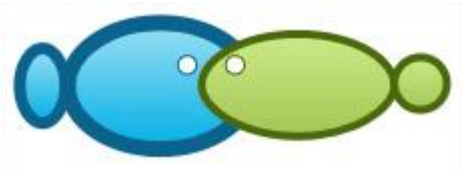

# *In vitro* transcription of dsRNA-LjRab7 - a tool for the development of interfering RNA as antiviral therapy in aquaculture of shrimp species

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**Abstract**. It is estimated that viral pathogens annually cause billion US dollar losses to the shrimp industry around the world. According to the World Organization for Animal Health (OIE), the main health problem facing the sector is the incidence of viral etiology diseases. Currently, RNAi-based therapeutic approaches have shown promise for the control of various types of viruses. The silencing of the endogenous Rab7 gene in crustaceans prevents the replication of various types of viruses that affect shrimps. The blocking of this gene inhibits both the infection of viruses with DNA, like WSSV, and also viruses with RNA (YHV, TSV, LSNV). Given this perspective, this study aimed to synthesize dsRNA-Rab7 by *in vitro* transcription. In this way, it was possible to obtain a 393 bp dsRNA corresponding to the Rab7 gene (GenBank AB379643.1) of *Penaeus japonicus* (LjRab7). The hybridization in the double-stranded structure was corroborated by analysis with RNase A. The implications of the study are discussed in the context of its importance as a tool for the development of methods for the control of viral pathogens associated with the aquaculture sector of penaeid shrimps.
**Key Words**: dsRNA, prawn, Rab7 gene, RNAi, transgene expression, virus.

**Introduction**. Nowadays, there is no therapeutic approach available for the control of viral pathogens in the shrimp farming industry. However, efforts are being made to develop antiviral therapies to combat these types of shrimp pathogens. The efforts are based primarily on the silencing of genes mediated by double-stranded RNA (dsRNA) or through mechanisms that involve the use of RNA interference (RNAi) (Saksmerprome et al 2009; Itsathitphaisarn et al 2017). It is reported that RNAi can protect shrimp from several highly pathogenic viruses including White Spot Syndrome Virus (WSSV) (Attasart et al 2009), Yellow Head Virus (YHV) (Tirasophon et al 2005, 2007), Taura Syndrome Virus (TSV) (Ongvarrasopone et al 2011), *Penaeus stylirostris* penstyldensovirus 1 (PstDV1), and *Penaeus monodon* Densovirus (PmDNV) (Attasart et al 2011; Saksmerprome et al 2013; Chimwai et al 2016).

RNAi-based mechanism has proven to be a promising prophylactic and therapeutic method for the management of viral diseases that affect shrimp. The mechanism of action of RNAi is initiated by a dsRNA molecule, which leads to the degradation of messenger RNA (mRNA) from specific and homologous sequences (Fire et al 1998). In shrimps, dsRNA complementary to a viral gene like the YHV protease has proven to be effective in preventing and/or curing infections caused by this virus in *P. monodon* (Yodmuang et al 2006; Tirasophon et al 2007). Furthermore, the effectiveness of an RNAi in suppressing

the replication of *Penaeus stylirostris* Densovirus (PstDNV) in *Penaeus vannamei* has been demonstrated both preventively and therapeutically (Ho et al 2011). Additionally, another approach is to use the RNAi relating to an endogenous gene needed for virus invasion. One such target is the intracellular traffic pathways used in the viral multiplication process. For example, the knockdown of Rab7 gene not only prevents WSSV replication, but also YHV (Ongvarrasopone et al 2008; Kongprajug et al 2017), Laent-Singh Virus (LSNV) (Ongvarrasopone et al 2010) and TSV (Ongvarrasopone et al 2011). This shows that Rab7 performs a fundamental role in the regulation of the intracellular trafficking of several types of viruses in shrimps. Given this perspective, this research aimed to establish a detailed procedure for the synthesis of RNAi (dsRNAi Rab7) by *in vitro* transcription, to use this tool in the development of procedures for the control of viral pathogens that affect the crustacean aquaculture industry.

**Material and Method**. RNA was isolated from the hepatopancreatic tissue of *Penaeus japonicus* (Kuruma shrimp), following the indications of the Nucleo-Spin RNA® kit (Macherey-Nagel). To verify the quality of the isolated RNA, the indications of the Agilent RNA 6000 Nano® kit were followed. To carry out cDNA synthesis by RT-PCR, the indications of the qScript® kit (Quanta Biosciences) were followed. The used reaction protocol was as follows: 1 µL of isolated RNA (1-10 µg of total RNA), 14 µL of nuclease-free water, 4 µL of qScript reaction mix (5x) and 1 µL of qScript reverse transcriptase, for a final volume of 20 µL. It was gently vortexed and then centrifuged for 10 s. Subsequently, it was placed in a thermal cycler under the following program: 1 cycle at 22ºC for 5 min, 1 cycle at 42ºC for 30 min, and 1 cycle at 85ºC for 5 min, with a stop at 4ºC. PCR amplification was carried out following the indications of the PCR Q5 kit, High-Fidelity DNA Polymerase® (New England Biolabs). A final reaction volume of 20 µL was obtained with 4 µL of reaction buffer Q5 (5x), 0.4 µL of dNTPs (10 mM), 1 µL of (10 mM) Rab7-F 5'-GGA TAC AGC TGG TCA AGA GAG ATT-3', 1 µL of (10 mM) Rab7-R 5- GGT CAA TTT GAT CTG GTC TGG AA-3', 1 µL of cDNA (5 ng/µL), 0.2 µL of high-fidelity DNA polymerase Q5 and 12.4 µL of free water nucleases. The thermal cycling program used was the following: initial denaturation at 98°C for 5 min, followed by 25 cycles at 98°C for 2 min, 98°C for 15 s, 60°C for 15 s, 72°C for 15 s and with a final extension cycle at 72°C for 2 min. The amplified product (393 bp, LjRab7, GenBank AB379643.1) was verified through electrophoresis (1% agarose, stained with Gel-Red, run for 1 h at 90 V, and using the Hyper Ladder™ II - Bioline - molecular weight marker). For PCR product purification, the indications of NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) were followed. The purified product (393 bp) was verified through electrophoresis (1% agarose, stained with Gel-Red, run for 1 h at 90 V, and using the Hyper Ladder™ II - Bioline - molecular weight marker).

Ligation was carried out following the indications of the CloneJET PCR Cloning® kit (Invitrogene, Thermo Scientific) and according to the following protocol: 5 µL of reaction buffer, 0.5 µL of PCR amplified product, 0.5 µL of vector pJET, 3.5 µL of nuclease-free water and 0.5 µL of T4 ligase, final reaction volume of 10 µL. The ligation mixture was incubated at room temperature (22°C) for 20 min, and then used in the transformation process. For transformation, 5 µL of the ligation mix were poured into 50 µL of frozen bacteria (*E. coli* Top10 chemically competent cells), vortexed, and incubated for 20 min on ice to then perform a heat shock at 42°C for 45 s, after which it was rapidly cooled on ice. 10 µL were spread onto plates with Luria-Bertani (LB) agar medium and incubated at 37ºC overnight.

Plasmid purification was carried out following the indications of the Plasmid DNA purification kit, following the NucleoSpin® Plasmid/Plasmid (NoLid) protocol. Then, aliquots of the samples (100-500 ng $\mu L^{-1}$) with a volume of 10 µL were mixed with 3 µL of the primer pJET-F and R. Once verified by sequencing analysis, a transformation was carried out with the plasmid pJET-LjRab7 in the forward and reverse directions (sense and antisense), proceeding in the same way as previously described, with the exception that the plasmid concentration used was 1 ng $\mu L^{-1}$. Subsequently, the bacteria (50 mL) were cultured in LB medium supplemented with ampicillin (50 µL, 100 µg $mL^{-1}$) overnight at 37°C and with shaking (200 rpm). To carry out the purification of plasmids pJET-LjRab7 (sense and antisense), the indications of the GeneJET Plasmid Midipreps (Invitrogene,

Thermo Scientific) kit were followed. Once the plasmid DNA had been purified, its concentration was quantified spectrophotometrically (260 nm).

The linearization of plasmids by restriction enzyme digestion was carried out following the indications of the *Xba*I commercial kit (Thermo Scientific), according to the following protocol: 10 µg of plasmid DNA, 10 µL of reaction buffer (1x Tango Buffer, 33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg $mL^{-1}$ BSA), 4 µL of restriction enzyme and completed with water until reaching 100 µL of total reaction volume. It was mixed gently, centrifuged at low speed for a few seconds, and incubated at 37°C for 4 h. At 3 hours after the incubation, a 5 µL aliquot was extracted from the reaction mixture and it was analyzed by electrophoresis (1% agarose, 110 V, 400 mA, 1 h, Hyper Ladder™ I - Bioline; molecular weight marker) to verify the presence of an expected 3367 bp band, pJET-LjRab7 (the length of plasmid pJET1.2/Blunt is 2974 bp, plus the insert of 393 bp).

Purification of plasmids pJET-LjRab7 (sense and antisense) was conducted following the indications of NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). *In vitro* transcription was carried out following the indications of the MEGAscript® kit (Ambion, Life technologies) and according to the next protocol. The entire transcription reaction was conducted at room temperature and mixing 2 µL of the ATP solution, 2 µL of CTP, 2 µL of GTP, 2 µL of UTP, 2 µL of the 10x reaction buffer, 1 µg of DNA, 2 µL of the enzyme mixture and water to make up the final volume of 20 µL. Then, it was mixed with the pipet, briefly centrifuged and incubated for 16 h at 37°C. Subsequently, 1 µL of Turbo DNase was added in order to eliminate the DNA remains; for this, it was mixed well and incubated for 15 min at 37ºC.

To recover the RNA, the lithium chloride precipitation method was used, for which 30 µL of nuclease-free water and 30 µL of the lithium chloride solution were added. It was mixed and cooled for 30 min at -20°C. Then, it was centrifuged at maximum speed for 15 min at 4°C. The supernatant was carefully removed. To wash the pellet, 1 mL of 70% ethanol was added and it was centrifuged again. Then, supernatant was discarded and resuspended in 20 µL of TE buffer (pH 8.0). The concentration was determined by spectrophotometry (260 nm) and finally stored at -20°C.

**Results**. After *in vitro* transcription, the synthesized single-stranded RNA (ssRNA) was heated to produce dsRNA, by incubating the ssRNA-LjRab7 mixture (in both orientations, sense and antisense, and concentration 1:1) at 70ºC for 15 min, and gradually reducing the temperature until reaching 22ºC. The final concentration of dsRNA-LjRab7 was determined by spectrophotometry at 260 nm. The result was verified through electrophoresis (1% agarose, stained with Gel-Red, run for 1 h at 90 V, and using the Hyper Ladder™ II - Bioline - molecular weight marker.

To verify the correct hybridization of the dsRNA, it was digested with RNase A (an enzyme that degrades ssRNA in the presence of a NaCl concentration ≥0.2 M). To do this, 0.5 µL of the synthesized RNA (ssRNA and dsRNA) were incubated for 15 min at 37ºC, in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and with 0.1 µL of the enzyme (10 mg $mL^{-1}$, 1/100) and 9.4 µL of NaCl solution (0.3 M), for a total reaction volume of 10 µL. The result was verified through electrophoresis (1% agarose, stained with Gel-Red, run for 1 h at 90 V, and using the Hyper Ladder™ II - Bioline - molecular weight marker).

Once the purification of the total RNA and the synthesis of cDNA by RT-PCR had been completed, the amplification of the 393 bp fragment corresponding to the Rab7 gene of the *P. japonicus* (LjRab7) was carried out. Subsequently, a purification procedure of the fragment obtained by PCR amplification was conducted, and the result was visualized by electrophoresis analysis (Figure 1A).

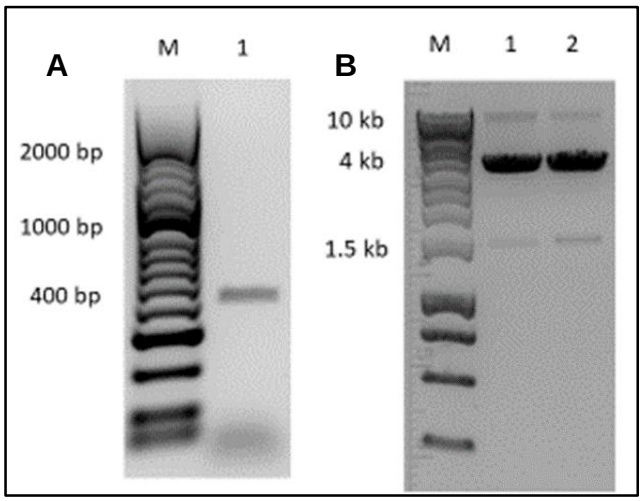


Figure 1. A - purified LjRab7 fragment (393 bp) amplified; B - pJET-LjRab7 plasmids (3367 bp) linearized by restriction; 1 - sense; 2 - antisense; M - molecular weight marker.

After having carried out both the ligation and transformation process (cloning), as well as the sequencing analysis to determine the orientation of the fragments linked to the vector (sense and antisense), we proceeded to conduct the purification process of the recombinant vectors in both orientations (sense and antisense). Subsequently, a digestion with the restriction enzyme *Xba*I was carried out in order to linearize the vectors. The results were visualized by electrophoresis (Figure 1B).

After linearizing the vectors in both orientations and having carried out the *in vitro* transcription process with both vectors, the result was visualized by electrophoresis (Figure 2A). Verification of the correct hybridization of the 393 bp fragments synthesized by *in vitro* transcription was done by digestion with RNase A, an enzyme that degrades ssRNA in the presence of a NaCl concentration ≥0.2 M. The result was visualized by electrophoresis (Figure 2B).

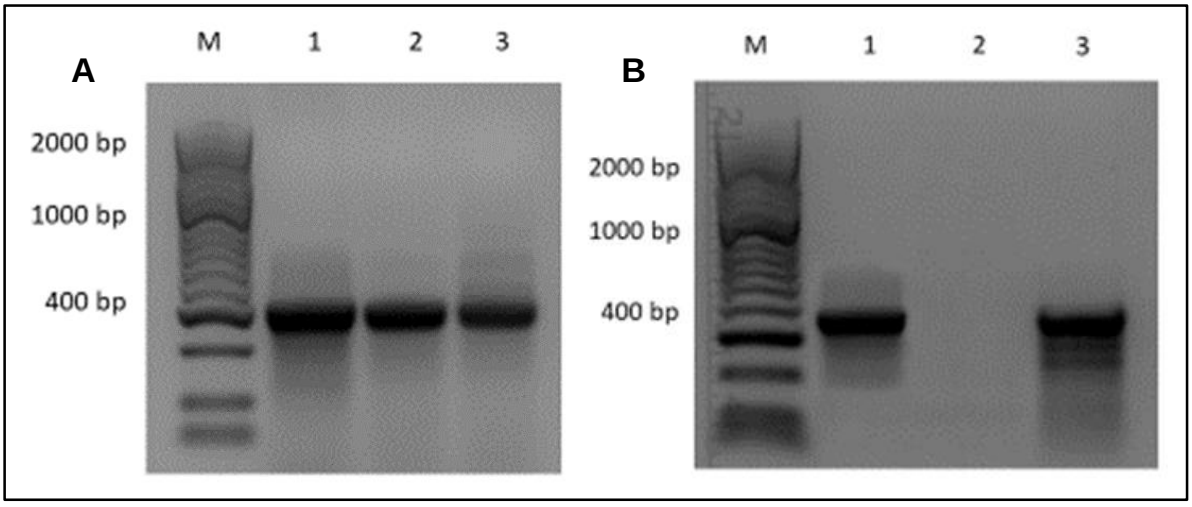


Figure 2. A - ssRNA-LjRab7 in both orientations; 1 - sense; 2 - antisense (393 bp); 3 - dsRNA-LjRab7 (393 bp); B - 1: ssRNA- LjRab7 untreated, 2: ssRNA- LjRab7 treated with Rnase A; 3: dsRNA- LjRab7 treated with Rnase A (393 bp); M - molecular weight marker.

**Discussion**. The development of this protocol constitutes a tool of interest in the context of the improvement of methods for the control of viral pathogens in the crustacean aquaculture sector. For example, the generated dsRNA (dsRNA-LjRab7) could be used in combination with other dsRNAs aimed at silencing other therapeutic targets, such as the

gene for the WSSV capsid protein (VP28) (Xu et al 2007; Sarathi et al 2008), the gene for YHV protease (Yodmuang et al 2006; Tirasophon et al 2007; Sinnuengnong et al 2018), a putative endogenous YHV receptor (Assavalapsakul et al 2006), or an early endosomal protein (PmEEA1) employed by YHV for the successful infection in *P. monodon* (Posiri et al 2019), constituting a gene therapy with a synergistic effect by combining the silencing of two or more genes simultaneously (Attasart et al 2011; Chimwai et al 2016; Chaimongkon et al 2020). The results obtained by Ongvarrasopone et al (2011) for TSV showed that both high (2.5 μg $g^{-1}$ shrimp) and low (0.625 μg $g^{-1}$ shrimp) doses of dsRNA-Rab7 can specifically knockdown LvRab7 (*P. vannamei* Rab7) by nearly 90%, as well as TSV multiplication by 90%. In addition, the high degree of conservation (>94%) of this sequence (GenBank AB379643.1, LjRab7) allows its effectiveness in several species of penaeid shrimps, including two of the main commercial species: *P. vannamei* (GenBank FJ811529.1; 94.01%) and *P. monodon* (GenBank HQ128578.1; 96.44%) (Ongvarrasopone et al 2011). Moreover, it should be noted that with the use of this approach, both preventive and prophylactic protection would be obtained (Ho et al 2011; Chimwai et al 2016).

Previous studies about the suppression (for 9 days) of PmRab7 by dsRNA-PmRab7 reported no effect on shrimp survival. Thus, it is most likely that Rab7 protein turn-over is fast in the cell after treatment is stopped (Ongvarrasopone et al 2008). As an endogenous gene, Rab7 can be rapidly recovered. Moreover, according to Ongvarrasopone et al (2011), low levels of PmRab7 expression in shrimp did not cause any type of morphological changes in test animals. According to Ongvarrasopone et al (2008), PmRab7 mRNA was almost totally inhibited (90%) on day 2 after injection. Nevertheless, the expression of PmRab7 mRNA remained at a relatively low level from day 2 to day 9 compared with the control. The inhibition effect of PmRab7 caused less than 5% shrimp mortality, which is very similar to the control groups.

Although RNAi-based therapeutic approaches have been very promising for the control of viral diseases in shrimp at the experimental level, the application of such technology at the field level remains a challenge because, so far, it has only been managed through injections, and this is not economically viable when working with millions of individuals. Similarly, dsRNA must be protected against environmental degradation by RNAse through an oral administration mechanism that allows this obstacle to be re-solved. The application of this technology in small-scale tests suggests the high efficiency of RNAi in the control of shrimp viruses that cause high mortality rates and slow growth syndrome (Saksmerprome et al 2009; Sinnuengnong et al 2018; Charoonnart et al 2019). Therefore, finding alternative dsRNA production and delivery systems that can be safely used in shrimp aquaculture is a key challenge for the future application of this technology.

**Conclusions**. This research has proven the feasibility of producing double-stranded RNA, that is the basis of the RNAi, that can be used for silencing genes that could impede the transmission or pathological consequences of viral infections. This could become an important tool for the development of methods for the control of viral pathogens associated with the aquaculture sector of penaeid shrimps.

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**Conflict of Interest**. The authors declare that there is no conflict of interest.

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