Journal of Visualized Experiments

Two-layered Membrane Sandwich Method for Laodelphax striatellus Saliva Collection

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62831R1
Full Title:	Two-layered Membrane Sandwich Method for Laodelphax striatellus Saliva Collection
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TITLE:

Two-layered Membrane Sandwich Method for *Laodelphax striatellus* Saliva Collection

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SUMMARY:

The present protocol describes a method to collect sufficient saliva from piercing-sucking insects using an artificial medium. This is a convenient method for collecting insect saliva and studying salivary function on insect feeding behavior and vector-borne virus transmission.

ABSTRACT:

Rice stripe virus (RSV), which causes significant economic loss of agriculture in East Asia, entirely depends on insect vectors for its effective transmission among host rice. *Laodelphax striatellus* (small brown planthopper, SBPH) is the primary insect vector that horizontally transmits RSV while sucking sap from the phloem. Saliva plays a significant role in insects' feeding behavior. A convenient method that will be useful for research on insects' saliva with piercing-sucking feeding behavior is described here. In this method, insects were allowed to feed on an artificial diet sandwiched between two stretched paraffin film layers. The diet containing the saliva was collected each day, filtered, and concentrated for further analysis. Finally, the quality of collected saliva was examined by protein staining and immunoblotting. This method was exemplified by detecting the presence of RSV and a mucin-like protein in the saliva of SBPH. These artificial feeding and saliva collection method will lay a foundation for further research on factors in insect saliva related to feeding behavior and virus transmission.

INTRODUCTION:

Rice stripe virus (RSV), a negative-stranded RNA virus in the genus *Tenuivirus*, causes severe diseases in rice production in East Asia[1–3]. Transmission of RSV from infected rice plants to

healthy ones depends on insect vectors, mainly *Laodelphax striatellus,* which transmits RSV in a persistent-propagative manner. SBPH acquires the virus after feeding on RSV-infected plants. Once inside the insect, RSV infects the midgut epithelial cell one day after feeding and then passes through the midgut barrier to penetrate the hemolymph. Subsequently, RSV spreads into different tissues *via* the hemolymph and then propagates. After a latent period of about 10–14 days post-acquisition, the virus inside the salivary gland can be transmitted to the healthy host plants *via* the secreted saliva while SBPH sucks sap from the phloem[4–10]. An efficient feeding process and various factors in the saliva are essential for the spread of RSV from the insect to the host plant.

Insect saliva secreted by salivary glands is believed to mediate insects, viruses, and host plants. Hemipteran insects usually produce two types of saliva: gelling saliva and watery saliva[11–13]. Gelling saliva is mainly secreted into the apoplasm to sustain the movement of the stylet among host cells and is also related to overcoming plant resistance and immune responses[14–17]. At the probing stage of feeding, insects intermittently secrete gelling saliva that immediately gets oxidized to form a surface flange. Then, single or branched sheaths encase the stylet to reserve a tubular channel[18–20]. The surface flange on the epidermis is presumed to facilitate penetration of the stylet by serving as an anchor point, while the sheaths around the stylet may provide mechanical stability and lubrication[16,21–23]. NIshp was identified as an essential protein for salivary sheath formation and successful feeding of brown planthopper (*Nilaparvata lugens*, BPH). Inhibition of the expression of the structural sheath protein (SHP) secreted by the aphid *Acyrthosiphon pisum* reduced its reproduction by disrupting feeding from host sieve tubes[24]. Moreover, in some insect species, gel saliva factors are supposed to trigger plant immune responses by forming so-called herbivore-associated molecular patterns (HAMPs). In *N. lugens*, NIMLP, a mucin-like protein related to sheath formation, induces plant defenses against feeding, including cell death, the expression of defense-related genes, and callose deposition [25,26]. Also, some gel saliva factors in aphids have been proved to trigger plant defense responses *via* gene-to-gene interactions similar to pathogen-associated molecular patterns[12,15,27].

For studying the saliva factors essential for insect feeding and/or pathogen transmission, it is necessary to analyze secreted saliva. Here, artificial feeding and collection methods to obtain sufficient amounts of saliva are described for further analysis. Using a medium containing only a single nutritional element, many saliva proteins were collected and analyzed by silver staining and western blotting. This method will be helpful in further research on factors in saliva that are essential for RSV transmission by SBPH.

PROTOCOL:

1. SBPH maintenance

1.1 Rear the viruliferous and RSV-free SBPH individuals in a glass incubator (65 x 200 mm) with 5–6 rice (*Oryza sativa* cv. Nipponbare) seedlings per glass chamber in the laboratory. Grow the rice plants at 25 °C under a 16 h light / 8 h dark photoperiod.

NOTE: The viruliferous and RSV-free SBPH individuals were initially caught in Jiangsu Province, China.

1.2 Detect RSV in SBPH by dot-enzyme-linked immunosorbent assay (dot-ELISA) with a rabbit RSV-specific polyclonal antibody (see **Table of Materials**) raised against the RSV ribonucleoproteins (RNPs).

NOTE: For ensuring high offspring infection efficiency, viruliferous females were maintained separately, and 15% of their offspring were randomly tested for RSV infection. The details of dot-ELISA are described in steps 1.3–1.7.

1.3 Homogenize single SBPH in 20 µL of coating buffer (0.05 M Na_2CO_3-$NaHCO_3$, pH 9.5). Spot 3 µL of each on nylon membrane (see **Table of Materials**), and then dry the membrane at room temperature (RT).

1.4 Incubate the membrane with 15 mL of blocking buffer (PBS + 3% skim milk) for 30 min at room temperature.

1.5 Incubate the membrane with diluted primary rabbit-antibodies against RSV (1:10000) in 15 mL of PBS for 1 h at RT and wash the membrane three times with PBS for 5 min incubation each time.

1.6 Incubate the membrane with 1.5 µL of horseradish peroxidase-conjugated goat anti-rabbit antibodies (see **Table of Materials**) in 15 mL of PBS and wash three times with PBS for 5 min incubation each time.

1.7 Develop the immunoblots with Enhanced HRP-DAB Chromogenic Kit (see **Table of Materials**) according to the protocols provided by the manufacturer.

2. Preparation of feeding chamber and artificial diet

2.1 Weigh 2 g of sucrose powder and dissolve it in 40 mL of ddH_2O to prepare 5% sucrose aqueous solution as the artificial diet.

2.2 Filter the solution through a 0.22 µm filter to remove bacterial contamination and impurities.

2.3 Starve 200 3^{rd}–5^{th} SBPH larvae for 3–5 h before introducing them into the chamber.

NOTE: 200 SBPH are prepared for one chamber; more SBPH should be prepared for several chambers.

2.4 Prepare the glass cylinders as feeding chambers (**Figure 1A**). Cover one open end of

the chamber with a paraffin membrane (see **Table of Materials**) before introducing the experimental insects.

NOTE: Each cylinder is 15.0 cm long and 2.5 cm in diameter. These glass cylinders are custom-made according to the experimental requirement.

2.5 Transfer insects into a glass cylinder.

2.6 Cover the other end of the chamber with stretched paraffin membrane (specifically, Parafilm M). Then, add 200 µL of artificial diet to it. Finally, cover the liquid with another layer of stretched paraffin membrane.

NOTE: The paraffin membrane is stretched to about double its original area.

2.7 Cover the chamber with aluminum foil, but leave the end with the artificial diet device exposed to the light.

3. Collection of SBPH saliva

3.1 Collect artificial diet from RSV-free and viruliferous SBPH separately at the end of 24 h period.

3.2 Cool the cylinder at 4 °C to immobilize the insects.

3.3 Uncover the outer film and collect the artificial diet liquid using a sterile pipette into 1.5 mL sterile tubes. Keep the collected saliva at -80 °C until analysis.

NOTE: The collected saliva could be stored at -80 °C for 1 year.

3.4 Rinse the inner membrane with 50 µL of fresh artificial diet three times by pipetting softly, and collect the artificial washing diet as described in step 3.3. Place the new artificial diet on the inner membrane and keep a freshly stretched Paraffin membrane on top.

3.5 Repeat steps 3.2 and 3.3 for 5 days to 2 weeks.

NOTE: Count the survival rate of artificial feeding SBPH and ensure sufficient supplement of fresh SBPH according to the survival rate.

3.6 Filter the collected samples through a 0.22 µm filter (see **Table of Materials**) unit to remove microbes and other contaminants.

4. Concentration of the collected saliva

4.1 Transfer the collected saliva samples in a 0.5 mL 10 kD centrifugal filter and spin at

5,500 x *g* at 4 °C for 20 min. Collect the supernatant and make the final volume to 100 µL.

4.2 Measure the concentration of the collected saliva using an appropriate UV-Vis spectrophotometer following steps 4.3–4.6.

4.3 Turn on the spectrophotometer and wash the pedestals three times with ddH_2O.

4.4 Select the following options on the screen in proper order: **Proteins | Protein A280 | Select Type | 1 Abs = 1 mg/mL**. Then, check the checkbox **Baseline Correction 340 nm**.

4.5 Load 2 µL of 5% sucrose aqueous solution as blank, touch **Blank** at the bottom of the screen.

4.6 After setting standards, load 2 µL of the collected saliva for measurement. Read and record the protein concentration.

NOTE: 1 mg of saliva proteins were finally collected in total at least.

5. Silver staining of saliva proteins

5.1 Extract protein from the insect saliva samples using sample loading buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 1% β-mercaptoethanol). Then, fractionate it by 10% SDS-PAGE (see **Table of Materials**). Load the 5% sucrose aqueous solution treated in the same manner as a negative control.

5.2 Load a 20 µL aliquot of the sample onto an SDS-PAGE gel alongside a prestained marker. Run the gel for 15 min at 90 Volt, and then 50 min at 140 Volt.

5.3 Fix the gel in 30% (vol/vol) ethanol, 10% (vol/vol) acetic acid for at least 30 min after electrophoresis.

5.4 Rinse the gel twice with 20% (vol/vol) ethanol and water separately for 10 min each time.

5.5 Sensitize the gel in 0.8 mM sodium thiosulfate for 1 min, and then rinse twice in water for 1 min each time.

5.6 Immerse the gel in 12 mM silver nitrate for at least 1 h, and then dip it in deionized water for 10 s before transferring it to the developer solution.

5.7 When the background of the gel is getting dark, immerse the gel in a stop solution (5% acetic acid) for at least 30 min to stop the reaction.

5.8 Wash the gel twice with water for 30 min each time. Develop the image with the

Detection System (see **Table of Materials**).

6. Protein detection by western blotting

6.1 Detect the saliva mucin-like protein of SBPH (LssgMP) and RSV by western blots using specific antibodies, respectively.

6.2 Treat insect saliva samples following step 5.1.

6.3 Load a 20 µL aliquot of the sample onto a 10% SDS-PAGE gel alongside a prestained marker and a 20 µL RSV non-infected saliva sample as a negative control. Run the gel for 15 min at 90 Volt, and then 50 min at 140 Volt.

6.4 Mix 100 mL of 10x protein transfer buffer (wet) with 900 mL of ddH_2O to a work solution (1x), and then transfer proteins to a polyvinylidene difluoride membrane using protein transfer buffer (1x).

6.5 Block the membrane in 5% skim milk with 0.01 M Tris-buffered saline with 0.05% Tween 20 (TBST) at room temperature (RT) for 1 h.

NOTE: In this protocol, mix 100 mL of 10x TBST with 900 mL of ddH_2O into the work solution.

6.6 Incubate the membrane with primary rabbit antibodies against RSV or LssgMP (both 1:10000) diluted in TBST at RT for at least 2 h.

NOTE: The production of primary antibodies against RSV was mentioned above. A biotechnology company produced the rabbit anti-LssgMP polyclonal antibody against LssgMP peptide GIQFDSYSASDLTRC.

6.7 Wash the membrane three times with TBST for 10 min of incubation each time.

6.8 Incubate the membrane with horseradish peroxidase-conjugated goat anti-rabbit antibodies diluted in 1:10000 TBST.

6.9 Develop the immunoblots with the enhanced chemiluminescence Western Blotting Detection System.

7. Detection of LssgMP expression pattern in SBPH

7.1 Immobilize the insects at 4 °C for 5 min.

7.2 Wash the insects with 75% ethanol and ddH_2O one by one, and then dissect the insects in pre-chilled TBS (0.01 M Tris-buffered saline).

7.3 Dissect the insects from the abdomen while severing the forelegs of SBPH at the coxa-trochanter joint by forceps; wash the midgut and the salivary glands twice in TBS to remove any contamination from the hemolymph.

7.4 Put five tissues into a 1.5 mL RNase-free tube to extract RNA. Consider each tube as one sample.

7.5 Perform RNA extraction according to the manufacturer's protocols and Reverse-transcriptional PCR (RT-PCR) (see **Table of Materials**).

7.6 Perform quantitative real-time PCR (qRT-PCR) to investigate the relative transcript expression levels of *LssgMP* in extracts of the whole body or various tissues of *L. striatellus*.

NOTE: The primer pairs used for gene amplification were *LssgMP*-q-F/*LssgMP*-q-R, SYBR Green-based qPCR was performed according to the manufacturer's protocol. The transcript level of *L. striatellus translation elongation factor 2* (*ef2*) was quantified with primer pair *ef2*-q-F/*ef2*-q-R for the normalization of the cDNA templates. And the primer sequences are attached below:*LssgMP*-q-F: TCCGACCTCACCAGAGTTTACAG; *LssgMP*-q-R: GCTTCGTCCCAGGTACTGATTCC; *ef2*-q-F: GTCTCCACGGATGGGCTTT; *ef2*-q-R: ATCTTGAATTTCTCGGCATACATTT.

REPRESENTATIVE RESULTS:

Schematics of artificial feeding installation and saliva collection

Figure 1A depicts the glass cylinder (15 cm x 2.5 cm) used as a feeding chamber to collect the saliva. Firstly, the SBPH larvae were starved for several hours to improve the collection efficiency and then immobilized by chilling for 5 min. After the insects were transferred into the glass cylinder, both open ends of the chamber were covered with stretched Paraffin membrane. At one end, 200 µL of 5% sucrose was sandwiched between two layers of Paraffin membrane extended to about double its original area (**Figure 1B**). The chamber was covered with foil, but the end with the artificial diet was exposed to light. Because SBPH displays phototropic behavior, the starved insects gathered at the end were exposed to light and fed on the artificial diet solution through the stretched inner Paraffin membrane. Based on it, saliva could be released into the artificial diet, which was collected each day. The Parafilm-diet device was replaced with a new one every day. In this way, the artificial diet was collected for 5 days to 2 weeks, and then the whole sample was concentrated to a final volume of 100 µL using a 10 KD centrifugal filter (**Figure 1C**). During the collection of saliva, the survival rate of SBPH feeding the 5% sucrose was counted. In the first 4 days, more than 80% SBPH survived. However, from the 5^{th} day onward, mortality increased quickly to 40%, and less than half SBPH survived on the 7^{th} day (**Figure 1D**). To collect sufficient saliva, fresh SBPH was suggested to supply on the 4^{th} day.

Verification of collected saliva proteins

For assessing the effectiveness of this collection method, the saliva sample was subjected to

protein analysis. Firstly, proteins were separated by SDS-PAGE, and then detected by silver staining (**Figure 2A**). Compared with the negative control (5% sucrose), the concentrated saliva samples from RSV-infected SBPH saliva contained many proteins that could be further analyzed, for example, by mass spectrometry. As the primary insect vector of RSV, SBPH transmits the virus to plants *via* its sucking-piercing feeding process. The successful release of RSV is related to saliva secretion, and RSV is considered to be an essential factor in viruliferous insects' saliva. Here, saliva was tested after collecting non-infected and viruliferous insects with an antibody against RSV and successfully detected the RSV coat protein (CP) in the viruliferous sample (**Figure 2B**). Another study found that mucin proteins are essential gel saliva proteins in hemipteran insects to meditate the formation of a sheath for feeding[23]. For confirming that SBPH also produces a mucin protein, the whole open reading frame of a putative mucin-encoding gene was amplified from RNA extracted from the saliva gland of SBPH. Its sequence was used as a query in BLAST analyses against the genome sequence of SBPH[28]. A gene consisting of 2175 bp, named *LssgMP,* was identified. An antibody against this protein was prepared previously and was used to detect the protein in the collected sample by western blot analysis. A 78 KD protein was detected in non-infected and viruliferous samples, demonstrating that LssgMP is a saliva protein (**Figure 2C**). Next, the transcript levels of *LssgMP* in different tissues (salivary gland, gut, and remaining body) were determined by qRT-PCR. The results showed that the gene transcript level was 20-fold higher in the salivary gland than in the gut and other body parts (**Figure 2D**), confirming the specific expression of *LssgMP* in the salivary gland.

FIGURE LEGENDS:

Figure 1: Diagram of feeding chamber with Paraffin membrane sandwich to allow feeding of SBPH on artificial diet. (**A**) Illustration of artificial feeding chamber. The cylinder is 15.0 cm long and 2.5 cm in diameter. At one end of the chamber is the Paraffin membrane sandwich; the other end and the cylinder wall covered with tin foil paper (slanted lines) represent the device covered with tin foil paper. The light source was set to attract SBPH to feed on the artificial diet. (**B**) Diagram of Paraffin sandwich containing artificial diet. 200 µL of 5% sucrose aqueous solution was sandwiched between two layers of Paraffin membranes stretched to about double its original area. (**C**) The concentration of collected saliva using a 10 KD centrifugal filter. (**D**) The survival rate of SBPH feeding on 5% sucrose. Mean and SEM was calculated from four biological replicates with three technical replicates.

Figure 2: Verification of collected saliva proteins. (**A**) Saliva proteins detection by silver staining. The 5% sucrose is negative control. (**B**) Detection of rice stripe virus coat protein (CP) in collected saliva by western blot analysis. (**C**) Western blotting to confirm the presence of LssgMP in collected saliva. (**D**) Specific expression of *LssgMP* in the salivary gland of *L. striatellus*. *ef2*: translation elongation factor 2 of SBPH. Mean and SEM was calculated from four biological replicates with three technical replicates.

DISCUSSION:

Successful rearing of insects on artificial diets was first reported in 1962 when Mittler and

Dadd described the Paraffin membrane technique to hold an artificial diet[29,30]. And this method has been explored in many aspects of insect biology and behavior, for example, nutrient supplement, dsRNA feeding, and virus acquisition. Based on the requirements of saliva analysis, 5% sucrose is used as the general artificial diet to collect saliva of SBPH in this study. For successful saliva collection, several critical steps are worth noting here. Firstly, starving the experimental insects before introducing them into the chamber is necessary to ensure the efficiency of saliva collection. Secondly, to imitate the stylet environment, a two-layer Paraffin sandwich is made. When SBPH feeds on the artificial diet, the salivary sheath forms at the inside end facing the diet, and the watery saliva is secreted subsequently. Thirdly, an artificial medium should be collected and exchanged in time to reduce microbial contamination in the collected sample. Finally, anesthetizing the insects at 4 °C for 5 min is essential to avoid the loss of insects while changing the artificial diet.

In contrast to most artificial diets which contain amino acids, vitamins, and carbohydrates, the advantages of 5% sucrose medium are noteworthy. First, it is easy to prepare. Second, the simple composition of the diet means few substances to interfere with further analysis of the various factors in saliva. Nevertheless, the survival ratio of the SBPH declined in the last days of the feeding period using 5% sucrose as the general artificial diet (**Figure 1D**). For overcoming this flaw, fresh SBPH should be supplied in time for enough saliva. And for further research of the salivary function on feeding behavior or virus transmission, the saliva collection duration should be limited to the accurate time before insects' mortality increased owing to innutrition. It seems to be a common limitation of the artificial medium. Some other studies found that the survival of *N. lugens* reared on the chemically defined diet D-97 was inferior to that of those raised on the susceptible rice variety TN1, implying that the original host supplies more than just food vector insects. And several studies have focused on optimizing chemically defined diets for continuous feeding of insects to improve the rearing efficiency.

Transcriptome analysis of the salivary gland is a traditional method for saliva protein identification. And different saliva proteins have been detected in the same species of *A. pisum* and *M. persicae*[14,31], and the abundance of saliva proteins determines the frequency of their detection[32]. However, a valid method to investigate the suspected protein in the saliva was lacking. This Paraffin membrane sandwich method provides an innovative manner to confirm a suspected saliva protein identified by transcriptome analysis, which is further proved by detecting LssgMP (**Figure 2C**). Moreover, protein analysis confirmed that abundant proteins are present in the collected saliva (**Figure 2A**), which is a sufficient quantity for further analysis by, for example, mass spectrometry. Proteomics analysis of collected saliva will be a direct and effective method for identifying secreted factors involved in the feeding stage of SBPH, minimizing the risk of detecting false-positive redundant proteins.

Saliva works as the virus carrier from insect to host plants and is an essential component of the vector-virus-plant interaction[14,33,34]. The titer of virus released from insect vectors is a crucial factor for the infection to the host. Compared with indirect methods that test the viral titer in infected plants, this protocol can directly detect RSV released by viruliferous SBPH

(**Figure 2B**). Supported by this Paraffin membrane sandwich method, further comparative analyses of saliva proteins collected from RSV-free and RSV-infected insects may also reveal potential candidates involved in the virus-plant-vector interaction.

DISCLOSURES:

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS:

This work was supported by the National Key Research and Development Program of China (No. 2019XFC1200503), by the National Science Foundation of China (No. 32072385), and by Youth Innovation Promotion Association CAS (2021084).

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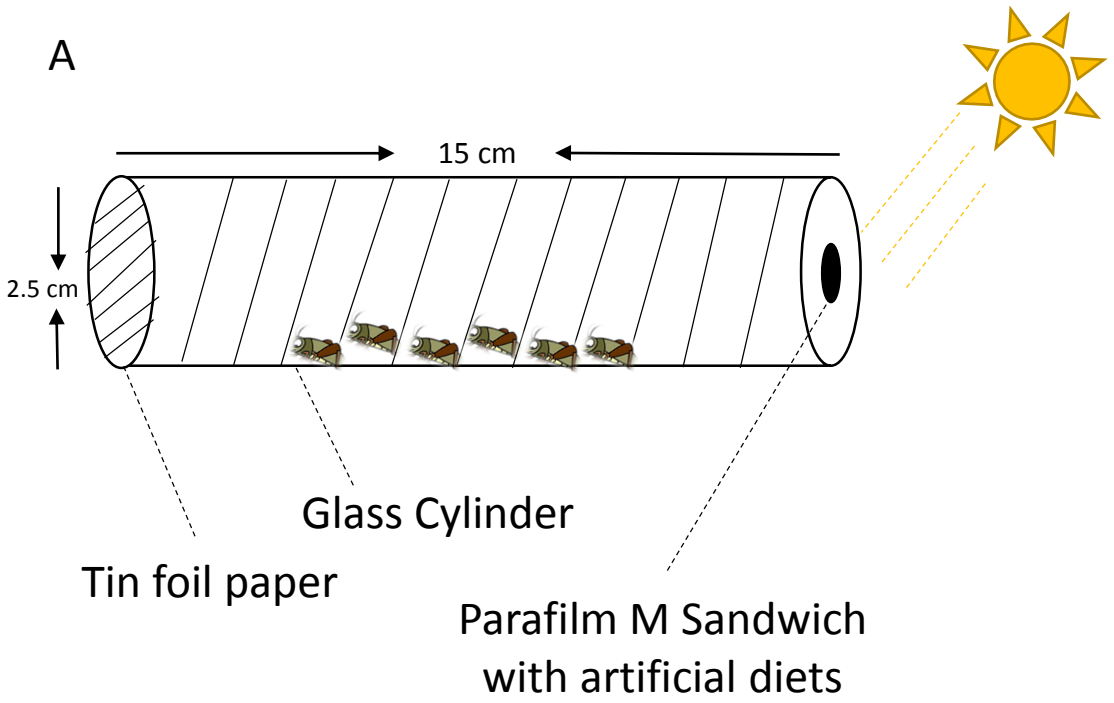
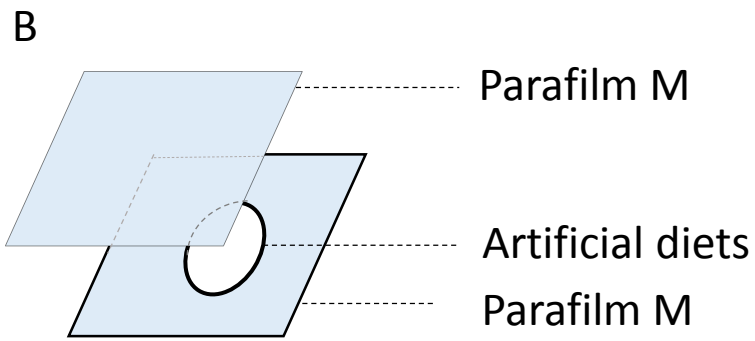
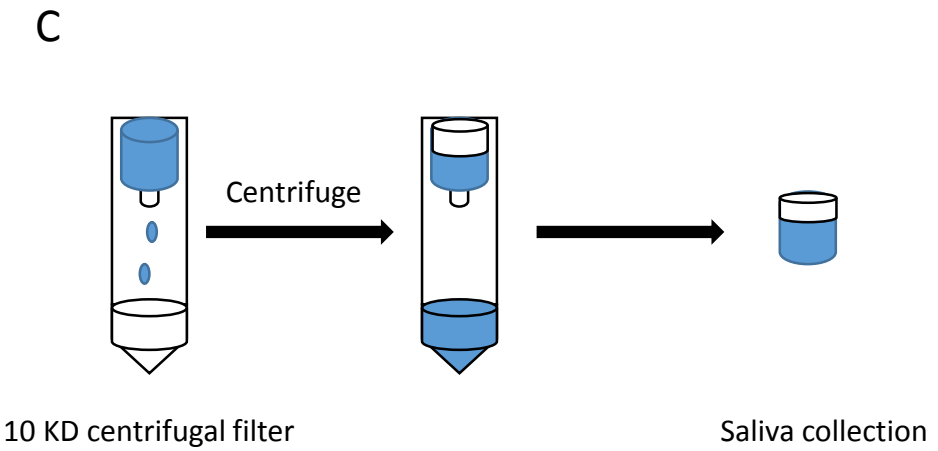
Figure 1







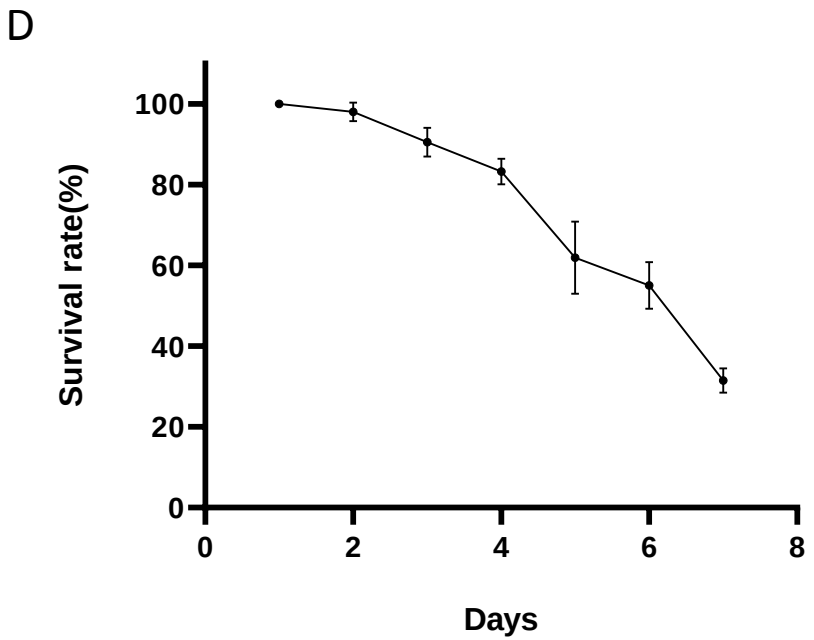
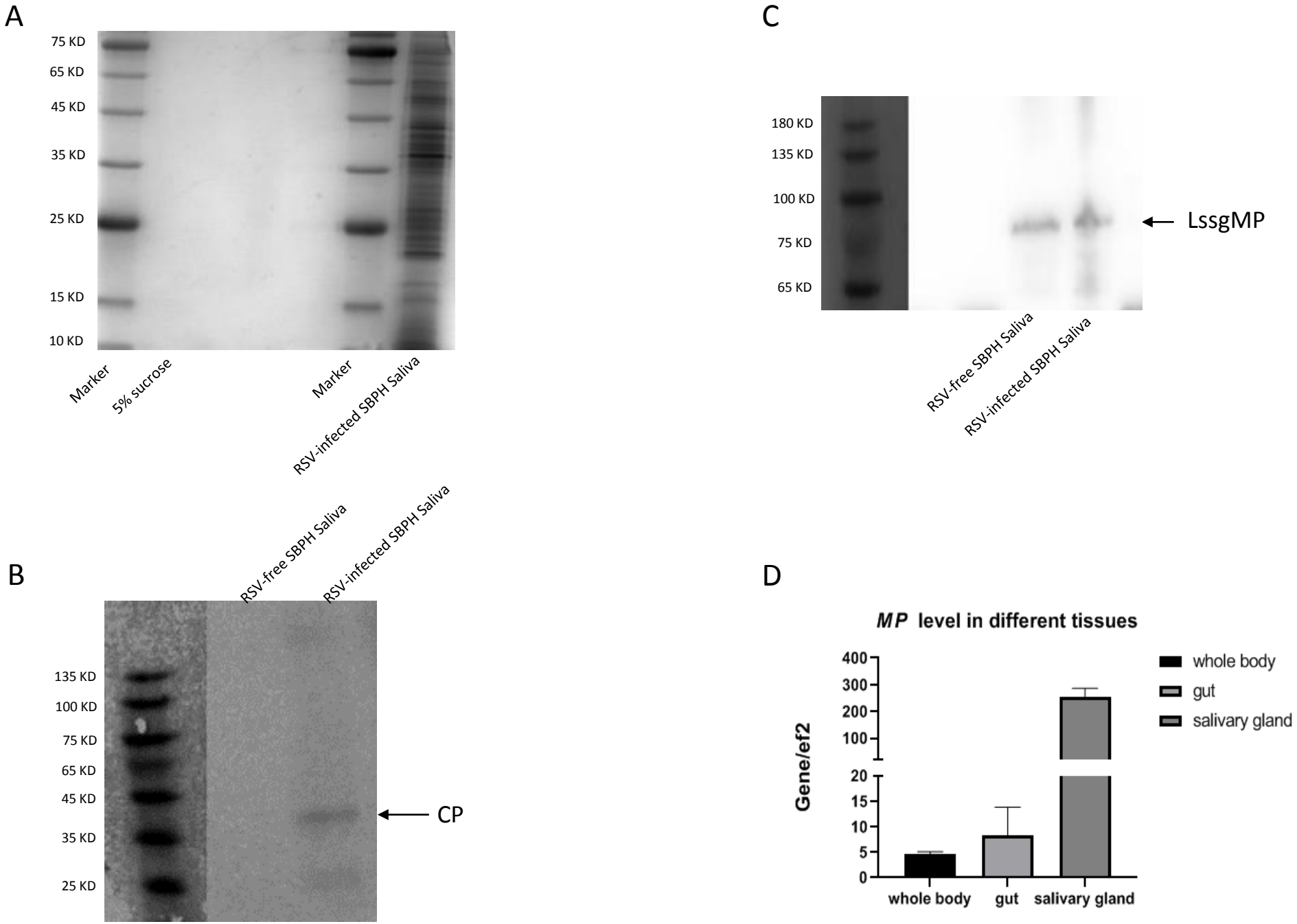


Figure2



Dear editor,

Thank you for the comments on our manuscript entitled "A Parafilm M membrane sandwich method to collect saliva of *Laodelphax striatellus*". We also thank the three reviewers for providing comments to help improve the manuscript. We have heeded all the specific comments, conducted suggested experiments whenever possible, included new data in the revised manuscript, and carefully proof-read the manuscript to minimize grammatical errors. Individual comments from editor and each reviewer are listed below, with our response directly following each comment.

Editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you and we have thoroughly proofread this manuscript and revised the spelling and grammar issues.

2. Please provide an institutional email address for each author.

We have edited the authors' email address as the institutional email addresses.

3. Please revise the following lines to avoid previously published work: 22-23, 69-72, 90-92, 157-159.

Thank you for your suggestion and we have reworded the sentences in line 22-23, 69-72, 90-92, 157-159 of previous manuscript. And sentences have been revised in the revised manuscript as follow:

Line 24-25: "Rice stripe virus (RSV), which causes great economic loss of agriculture in East Asia, completely depends on insect vectors for its effective transmission among host rice."

Line 69-72: "Also, some gel saliva factors in aphids had been proved to trigger plant defense responses via gene-to-gene interactions in a similar manner to pathogen-associated molecular patterns."

Line 83-86: "The viruliferous and RSV-free SBPH individuals were originally caught in Jiangsu Province, China, and were reared in a glass incubator (Φ65 × 200 mm) with five to six rice (Oryza sativa cv. Nipponbare) seedlings per glass chamber in our laboratory. Grow the rice plants at 25°C under a 16-h light/8-h

dark photoperiod."

Line 184-188: "6.5 Block the membrane in 5% skim milk with 0.01M Tris buffered saline with 0.05% Tween 20 (TBST) at room temperature (RT) for 1h. In this protocol, mix 100 ml 10x TBST with 900 ml ddH_2O to the work solution.

6.6Incubate the membrane in TBST diluted with primary rabbit antibodies against RSV or LssgMP (both 1:10000) at RT for at least 2h."

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

At the editor's request, we have rephrased the summary as follow (line 19-22):
"Here we describe a method to collect sufficient saliva from piercing-sucking insects using the artificial medium. This is a convenient method to collect insect saliva and will benefit further study of salivary function on insect feeding behavior and on vector-borne virus transmission. "

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text has been checked to avoid the personal pronouns.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: Amicon, Merck, Millipore, Parafilm M, Sigma, GE Healthcare, etc.

We have removed all the commercial language from the manuscript and the commercial products used in this work have been summarized in the Table of Materials

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the

imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have reworded the protocol section to meet the requirement (line82-216).

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We apologize for the missing details of the protocol of and have fulfilled details of the protocol steps. Detail is reworded in section of protocol (line106-154).

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

The protocol (line82-216) has been simplified to meet the requirement.

10. Line 88-92: Please mention how was the ELISA performed. Was a commercial kit used?

We apologize for the absent introduction of dot-enzyme-linked immunosorbent assay (dot-ELISA). The detail information of dot-ELISA has been mentioned in line87-105 in the revised text.

"1.2 Detect RSV in SBPH by dot-enzyme-linked immunosorbent assay (dot-ELISA) with a rabbit RSV-specific polyclonal antibody which was raised against the RSV ribonucleoproteins (RNPs).

(Note: To ensure high offspring infection efficiency, viruliferous females were maintained separately, and 15% of their offspring were tested for RSV infection randomly. The details of dot-ELISA is described in 1.3-1.7)

1.3 Homogenize single SBPH in 20 μL coating buffer (0.05 M Na_2CO_3-$NaHCO_3$, PH 9.5). Spot 3 μL of each on nylon membrane, and then dry the membrane at room temperature (RT).

1.4 Incubate the membrane into 15 ml blocking buffer (PBS+3% skim milk) for

30min at room temperature.
1.5 Incubate the membrane into 15 ml PBS with diluted primary rabbit-antibodies against RSV (1:10000) for 1h at RT and wash the membrane three times with PBS, for 5 min each time.
1.6 Incubate the membrane into 15 ml PBS with 1.5 μL horseradish peroxidase-conjugated goat anti-rabbit antibodies, and wash three times with PBS, for 5 min each time.
1.7 Develop the immunoblots with Enhanced HRP-DAB Chromogenic Kit according to the protocols provided by the manufacturer."

11. Line 94: How is the solution prepared? How much is prepared?

We have described the solution preparation in line107-108 of revised text.

"Weigh 2 g of sucrose powder and dissolved it in 40 ml ddH_2O to prepare 5% sucrose aqueous solution as the artificial diet".

12. Line 97-98: Please mention how many insects are transferred.

We transferred 200 3^{rd}-5^{th} instar insects into a chamber and the detail has been mentioned in line 111-114 of the revised text.

"2.3. Starve 200 3^{rd}-5^{th} SBPH larvae for 3–5 h before introducing them into the chamber.
(Note: 200 SBPH are prepared for one chamber, more SBPH should be prepared for several chambers.)"

13. Line 128-131/151-164: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings.

Thank you for your comment and we have reworded this part with step numbers in line 141-154/174-196 of revised manuscript.

14. Line 128-131: Please elaborate on the concentration method used in this study. Please include the details of the parameters and conditions necessary for performing this step.

Protein concertation was tested directly utilizing the NanoDrop one and we have added the testing method in line143-154 of the revised text.

"4.2 Measure the concentration of the collected saliva using NanoDrop One following steps 4.3-4.6.
4.3 Turn on the NanoDrop One and wash the pedestals with ddH_2O for three 3 times.

4.4 Select the following options on the screen in proper order: “Proteins” – “Protein A280” – “select type” – “1 Abs= 1 mg/ml”. Then check the checkbox “Baseline correction 340 nm”
4.5 Load 2 μL 5% sucrose aqueous solution as blank, touch “Blank” on the bottom of screen.
4.6 After setting standards, load 2 μL saliva collections for measurement. Read and record the protein concentration.
(Note: 1mg saliva proteins were finally collected in total at least.)”

15. Line 146-147: Please mention how the appropriate degree of staining is confirmed.

We have revised this part in line170-171:

“When the background of gel is getting dark, immerse the gel in stop solution (5% acetic acid) for at least 30 min to stop the reaction.”

16. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have revised the protocol at the request in line82-216.

17. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

We have checked and revised the figure and table according to the request.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

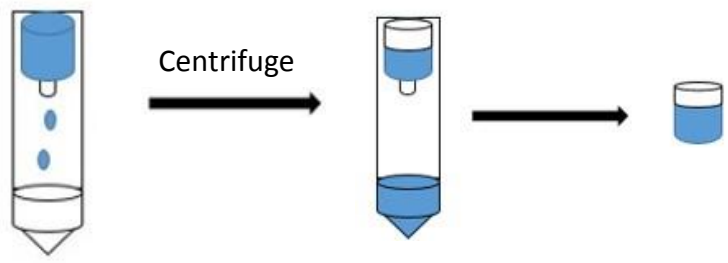
e) Any future applications of the technique

We have revised the discussion according to editor's request in which we have discussed the critical steps in the 1st paragraph (line288-302), modifications of technique in the 2nd paragraph (line303-306), limitation of this technique in the 2nd paragraph (line307-317), the significance with respect to existing methods and future applications of this technique in the 3rd and 4th paragraphs (line 318-338).

19. Figure 1: Please maintain a single space between the numeral and the unit (e.g., 10 KD).

The format has been revised as below.

C



10 KD centrifugal filter Saliva collection

20. Figure 2: Please provide the details of sample number and the statistical analysis performed to validate the results.

We apologize for missing the information. The sample number and statistical analysis have been added in the figure legend (line280-286).

"Figure 2. Verification of collected saliva proteins

(A) Saliva proteins detection by silver staining. The 5% sucrose is negative control. (B) Detection of rice stripe virus coat protein (CP) in collected saliva by western blot analysis. (C) Western blotting to confirm the presence of LssgMP in collected saliva. (D) Specific expression of *LssgMP* in salivary gland of *L. striatellus*. *ef2*: translation elongation factor 2 of SBPH. Mean and SEM were

calculated from four biological replicates with three technical replicates."

21. Please ensure that the Table of Materials includes all the essential supplies, chemical and reagents used in the study. Please sort the table in alphabetical order.

The table has included all information needed in this study and the table is resorted.

Reviewers' comments:

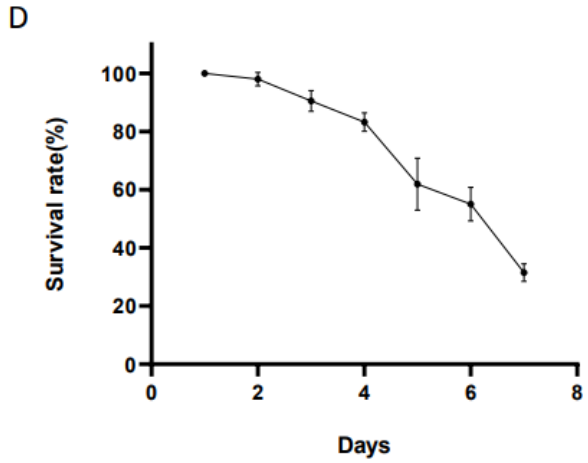
Reviewer #1:

The manuscript entitled "A Parafilm M membrane sandwich method to collect saliva of Laodelphax striatellus" describe a convenient method that used for the saliva collection of insects with piercing-sucking feeding behavior. It is a very conventional method that used for collecting insect saliva, and introduce this method by JoVE Produced Video will help new researches to follow.

I recommend to publish this protocol after the author adding the survival rate of SBPH rearing on the 5% sucrose, as this result was mentioned in the manuscript without any data.

We thank the reviewer for the positive assessment and suggestion for our work. In the revised manuscript, we have counted survival ration of SBPH feeding on artificial medium for 7 days. And the result has been described in the first section of result in line 233-237. And the figure is added as Fig1D with the figure legend in line276-278.

Line233-237："During collection of saliva, the survival rate of SBPH feeding the 5% sucrose was counted. In the first 4 days, more than 80% SBPH survived. However, from 5th day on, mortality increased quickly to 40%, and less than half SBPH survived on the 7th day (Fig.1D). To collect sufficient saliva, fresh SBPH were suggested supplied on the 4th day."



Line 276-278: "(D) Survival rate of SBPH feeding on 5% sucrose. Mean and SEM were calculated from four biological replicates with three technical replicates.

Reviewer #2:

Manuscript Summary:

The authors describe a convenient method that should be useful for research on the saliva of insects with piercing-sucking feeding behavior. In this method, insects were allowed to feed on an artificial diet solution sandwiched between two layers of stretched Parafilm M membrane. The food source (5% sucrose) containing the saliva was collected each day, filtered, and concentrated for further analysis. The quality of collected saliva was examined by protein staining and immuno-blotting. This method was used for detecting the presence of rice stripe virus (RSV) and an identified mucin protein in the saliva of small brown planthopper (SBPH).

Major Concerns:

Line 33- "a marked saliva protein in the saliva of SBPH" could be better stated.

Thank you for the suggestion and, we have reworded “a marked saliva protein” to “a mucin-like protein” (line35) in the revised text to express our meaning more clearly.

Line 43 - I think it also would be helpful to include the incubation period for RSV in SBPH. What is the period of time between insect acquisition of RSV and it's detection in salivary glands and saliva?

It is a good suggestion. And at the reviewer’s request, we have revised this part containing the detail time of acquisition and incubation (line42-48) as follow:

“SBPH acquires virus after feeding on RSV-infected plants. Once inside the insect, RSV infects the midgut epithelial cell in one day after feeding and then passes through the midgut barrier to penetrate into the hemolymph. Subsequently, RSV spreads into different tissues *via* the hemolymph and then propagates. After a latent period of about 10-14 days post acquisition, virus inside the salivary gland can be transmitted to healthy host plants via the secreted saliva while SBPH sucks sap from the phloem”.

Line 89 - should be " immunosorbent"

We apologize for the spelling error and this letter has been corrected (line87).

Line 89- 92.Authors might state briefly the working dilution, the source of RSV-specific polyclonal antibody (ie. mouse, rabbit, etc.) and if the antibody is commercially available

This detail protocol of dot-ELISA has been reworded in line87-105 of the revised text which contains detail information of RSV- specific polyclonal antibody (line88-89/98-99).

“1.2 Detect RSV in SBPH by dot-enzyme-linked immunosorbent assay (dot-ELISA) with a rabbit RSV-specific polyclonal antibody which was raised against the RSV ribonucleoproteins (RNPs).

(Note: To ensure high offspring infection efficiency, viruliferous females were

maintained separately, and 15% of their offspring were tested for RSV infection randomly. The details of dot-ELISA is described in 1.3-1.7)
1.3 Homogenize single SBPH in 20 μL coating buffer (0.05 M Na_2CO_3-$NaHCO_3$, PH 9.5). Spot 3 μL of each on nylon membrane, and then dry the membrane at room temperature (RT).
1.4 Incubate the membrane into 15 ml blocking buffer (PBS+3% skim milk) for 30 min at room temperature.
1.5 Incubate the membrane into 15 ml PBS with diluted primary rabbit-antibodies against RSV (1:10000) for 1h at RT and wash the membrane three times with PBS, for 5min each time.
1.6 Incubate the membrane into 15 ml PBS with 1.5 μL horseradish peroxidase-conjugated goat anti-rabbit antibodies, and wash three times with PBS, for 5 min each time.
1.7 Develop the immunoblots with Enhanced HRP-DAB Chromogenic Kit according to the protocols provided by the manufacturer."

Line 90- define "RNPs "
"RNPs" means "ribonucleoproteins", which has been defined in line89.

Line 110- 111- add "artificial" as in artificial diet or food source.
This word has been added in line 126.

Line 116 - " liquid " should be liquid (drop bold lettering)
We are sorry for the error format and it has been corrected (line129).

Line 123 - "new insects" is vague. Does this mean new SBPH or other insect species? The latter could add variability that affects the protocol and results.
We apologize for the confused description, and we meant "fresh SBPH" here. We have mentioned it in line 136-137.

Line 128-129 - "Concentrate the collected saliva samples using an appropriate concentration
method." Could be deleted, it is redundant
This sentence has been deleted according to the reviewer's comment.

Line 131 - "1 mg saliva proteins was collected in total " mention here measurement used to determine amount of protein
We apologize for the missing measurement. Protein concertation was tested directly utilizing the NanoDrop one and we have added the testing method in line143-154 of the revised text.

"4.2 Measure the concentration of the collected saliva using NanoDrop One following steps 4.3-4.6.
4.3 Turn on the NanoDrop One and wash the pedestals with ddH_2O for three 3

times.
4.4 Select the following options on the screen in proper order: “Proteins” – “Protein A280” – “select type” – “1 Abs= 1 mg/ml”. Then check the checkbox “Baseline correction 340 nm”
4.5 Load 2 μL 5% sucrose aqueous solution as blank, touch “Blank” on the bottom of screen.
4.6 After setting standards, load 2 μL saliva collections for measurement. Read and record the protein concentration.
(Note: 1mg saliva proteins were finally collected in total at least.)”

Line 137 - Please specify the percentage of acrylamide gel used for the separation of the proteins.
The percentage of acrylamide gel has been added in line158.
“Then fractionate it by 10% SDS-PAGE”

Line 151 - delete sentence (somewhat vague)
This sentence has been deleted according to the reviewer’s comment.

Line 152- define LssgMP
LssgMP means “the saliva mucin-like protein of SBPH”, and it has been added in line175.

Line 153 should be "50mM"
This part has been reworded (line156-157).

Line 153 - 155 - Would suggest rewording this section
We have reworded this section in line175-196 as follow：
“6.1 Detect the saliva mucin-like protein of SBPH (LssgMP) and RSV by Western blots using specific antibodies respectively.
6.2 Treat insect saliva samples following step 5.1.
6.3 Load a 20 μL aliquot of the sample onto an SDS-PAGE gel alongside a prestained marker and a 20 μL RSV non-infected saliva sample as negative control. Run the gel for 15 min at 90 Volt and then 50 min at 140 Volt.
6.4 Mix 100 ml 10x protein transfer buffer (wet) with 900 ml ddH2O to a work solution (1x), then transfer proteins to a polyvinylidene difluoride membrane using protein transfer buffer (1x).
6.5 Block the membrane in 5% skim milk with 0.01M Tris buffered saline with 0.05% Tween 20 (TBST) at room temperature (RT) for 1h. In this protocol, mix 100 ml 10x TBST with 900 ml ddH_2O to the work solution.
6.6 Incubate the membrane in TBST diluted with primary rabbit antibodies against RSV or LssgMP (both 1:10000) at RT for at least 2h.
(Note: The production of primary antibodies against RSV was mentioned above. The rabbit anti-LssgMP polyclonal antibodies against LssgMP peptide GIQFDSYSASDLTRC was produced by biotechnology company.)

6.7 Wash the membrane three times with TBST for 10 min each time.
6.8 Incubate the membrane in TBST with 1:10000 diluted horseradish peroxidase-conjugated goat anti-rabbit antibodies.
6.9 Develop the immunoblots with the enhanced chemiluminescence Western Blotting Detection System."

Line 157 - add concentration of Tris buffer and Tween 20
The concentration of Tris buffer and tween 20 has been added in step 6.5 (line184-186) and is described as follow:
"6.5 Block the membrane in 5% skim milk with 0.01 M Tris buffered saline with 0.05% Tween 20 (TBST) at room temperature (RT) for 1h. In this protocol, mix 100 ml 10x TBST with 900 ml ddH_2O to the work solution."

Line 159- mention briefly the type, the 'work dilution and source of LssgMP-antibody and if it is commercially available"
The detail information of antibody has been provide in protocol 6.6 from line187 to 191 and is described as follow:
"6.6 Incubate the membrane in TBST diluted with primary rabbit antibodies against RSV or LssgMP (1:10000) at RT for at least 2h.
(Note: The production of primary antibodies against RSV was mentioned above while the rabbit anti-LssgMP polyclonal antibody against LssgMP peptideGIQFDSYSASDLTRC was produced by biotechnology company.) "

Line 175- add "artificial" as in artificial diet or food source.
This word has been added in line 228.

Line 178- add "artificial" as in artificial diet or food source.
This word has been added in line 231.

Line 182 -delete second "collected"
This second "collected" has been deleted in line 239.

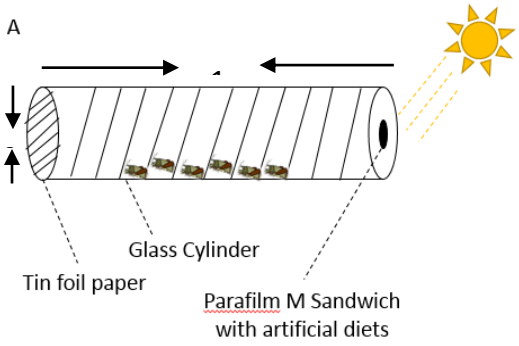
Line 200- replace "healthy" with non-infected
This letter has been replaced according to reviewer's comment in line 257.

Lines 225-240 some of this has been previously stated.
The Discussion is redundant, please consolidate.
Thank you for your comment and we have simplified and reworded the discussion at the request (line288-338). Critical steps, modifications of technique, limitation of this technique, the significance with respect to existing methods and future applications of this technique are covered in this revised discussion.

Figure 1A could be more descriptive….cylinder size? Any significance to the slanted

lines in the cylinder? Purpose of the orange colored icon?

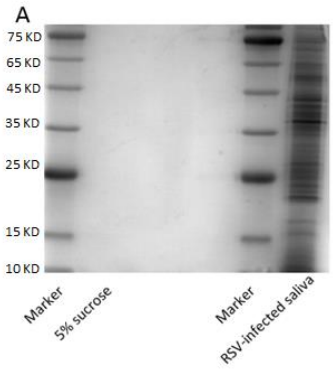
We have added more information in the figure 1A and reworded the figure legend to provide detail information of the figure (line269-273). Revised figure and figure legend are described as follow:

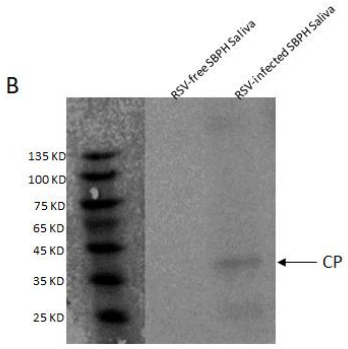


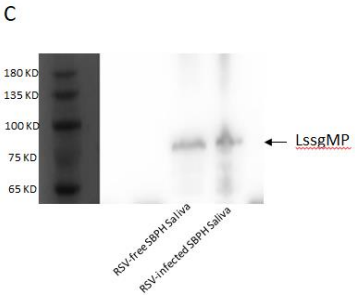
"(A) Illustration of artificial feeding chamber. The cylinder is 15.0 cm long and 2.5 cm in diameter. At one end of chamber is the Parafilm membrane sandwich, the other end, as well as the cylinder wall is covered with tin foil paper (slanted lines) represent the device is covered with tin foil paper. Set the light source to attract SBPH to feeding the artificial diet."

Figure 2A and 2C.....what are the size markers?

We apologized for the missing information of this figure. The Figure2 has been revised with the protein markers.







Reviewer #3:

Manuscript Summary:
Authors have described the widely used feeding method for collecting saliva of many hemipterans using 5% sucrose solution and a parafilm sandwich feeding approach. To confirm the secretion of saliva into the artificial diet, the authors have performed SDS-PAGE gel separation and Western blot analysis of the saliva proteins.

Major Concerns:
Since this is a methods paper, more specific details need to be provided for a new person to follow the protocol. For example, a person working with hoppers for the first time, need to know how to harvest the insects from the colony to perform the feeding bioassays. Also, other details such as protein quantification, dissections, RT-qPCR are missing. As the results includes the outcome of these techniques, respective methodology should be included in the methods to make the manuscript complete. Please find some examples of such missing details in more specific detail below.
Thanks for the critical suggestion, and this will improve the reference value of our work. At the reviewer's request, in the revised text, we have provided detail protocols of dot-ELISA (line 87-105), insect dissection (line 198-204) and RT-qPCR (line 205-216). Moreover, protein detection method (line155-196) and solution preparation (line107-108) have also been described in detail.

Although, the submitted manuscript is valuable to the entomology community, it is missing a lot of details as per the standard of Jove and needs to be extensively revised to make it more comprehensive for a novice researcher.
We apologize for the missing information in protocol. And the protocol section has be revised with detail information to meet the standard of JOVE.

Specific comments and minor concerns:
Line 33: Abstract: Please elaborate on the marked saliva protein? Is it a protein marker or a labeled protein?
We apologize for the confused description and we meant a new identified saliva protein (mucin-like protein) here. And in the revised manuscript, "a marked saliva protein" has been reworded to "a mucin-like protein" in line 35.

Line 37: Italicize genus "Tenuivirus"
The format has been changed in line 39

Line 40: Delete period after the parentheses
The period has been deleted (line 42).

Line 89: "Immunosorbent"
We apologize for the spelling error and this letter has been corrected in line 87.

Line 117: Were both the membranes removed or only the top membrane of the

sandwich layer was removed?

Only the top membrane of sandwich layer was removed. And to avoid this confusion, we have revised the sentences in line131-134:

"3.4. Rinse the inner membrane with 50 μl fresh artificial diet for 3 times by pipetting softly, and collect the washing artificial diet as described in step 3.3. Place new artificial diet on the inner membrane and then a fresh stretched Parafilm M on the top."

Line 131: How was the protein quantified?

Protein concertation was tested directly utilizing the NanoDrop one and we have added the testing method in line143-154 of revised text.

"4.2 Measure the concentration of the collected saliva using NanoDrop One following steps 4.3-4.6.

4.3 Turn on the NanoDrop One and wash the pedestals with ddH_2O for three 3 times.

4.4 Select the following options on the screen in proper order: "Proteins" – "Protein A280" – "select type" – "1 Abs= 1 mg/ml". Then check the checkbox "Baseline correction 340 nm"

4.5 Load 2 μL 5% sucrose aqueous solution as blank, touch "Blank" on the bottom of screen.

4.6 After setting standards, load 2 μL saliva collections for measurement. Read and record the protein concentration.

(Note: 1mg saliva proteins were finally collected in total at least.)"

Line 135: Was the negative control treated the same way as the positive control?

Yes. We have revised the sentence to avoid this confusion in line 156-159 as follow:

"Extract protein from the insect saliva samples using sample loading buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue and 1% β-mercaptoethanol). Then fractionate it by 10% SDS-PAGE. Load the 5% sucrose aqueous solution treated with the same manner as a negative control."

Line 152: LssgMP and rationale for using this protein as a marker. More detail about the primary antibody needs to be provided

The detail information of antibody has been provided in line187-191 and is described as follow:

"6.6 Incubate the membrane in TBST diluted with primary rabbit antibodies against RSV or LssgMP (both 1:10000) at RT for at least 2h.

(Note: The production of primary antibody against RSV was mentioned above. The rabbit anti-LssgMP polyclonal antibody against LssgMP peptide GIQFDSYSASDLTRC was produced by biotechnology company.)"

Line 158: Percent tween 20 used?

The percent of tween 20 has been added in protocol 6.5 (line 184-186) and is

described as follow:

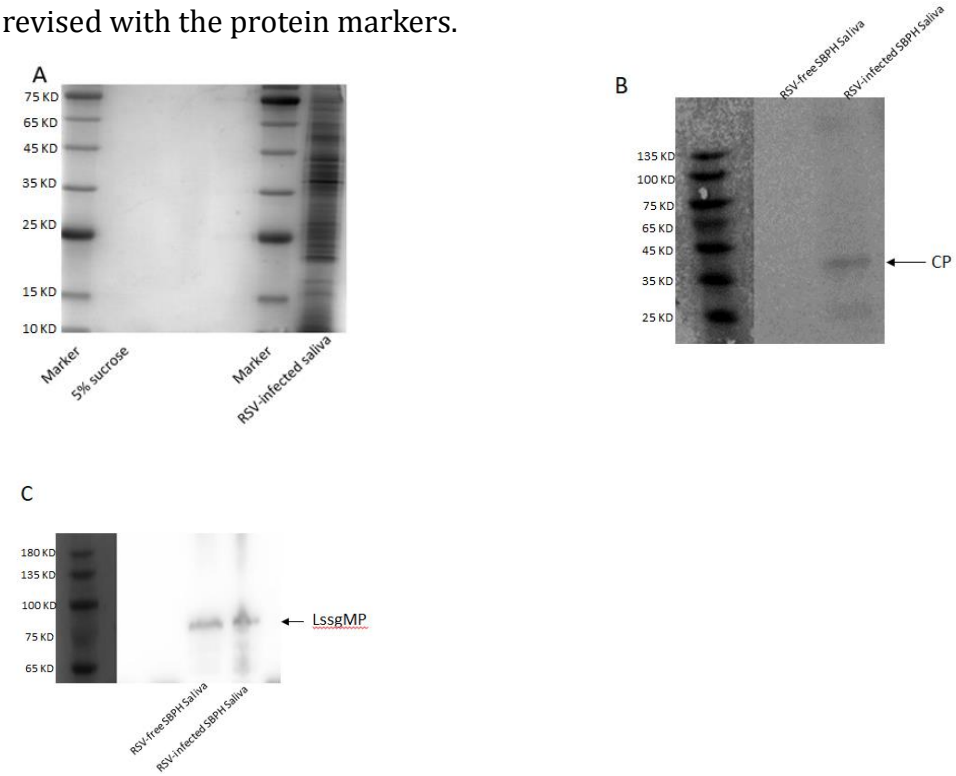
"6.5 Block the membrane in 5% skim milk with 0.01M Tris buffered saline with 0.05% Tween 20 (TBST) at room temperature (RT) for 1h. In this protocol, mix 100 ml 10x TBST with 900 ml ddH_2O to the work solution. "

Line 181: None of the methodology described in the results section was mentioned under the methods section. Please include these respective details in the methods.

We apologize for the missing detail method for this section. And in the revised manuscript we have provided detail protocol of insect dissection(line 198-204), RT-qPCR (line 205-216)， sliver staining(line 156-173) and western blotting(line 175-196).

Figure 2: Please include marker for the western blot detecting the presence of the virus and the sheath protein.

We apologized for the missing information of this figure. The Figure2 has been revised with the protein markers.



We appreciate the comments from all the reviewers to improve the manuscript.

We hope that the current paper is now suitable for publication in JoVE

Sincerely yours,

Yan Huo