Journal of Visualized Experiments

Detecting virus and salivary proteins of a leafhopper vector in the plant host

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63020R2
Full Title:	Detecting virus and salivary proteins of a leafhopper vector in the plant host
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TITLE:

Detecting Virus and Salivary Proteins of a Leafhopper Vector in the Plant Host

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SUMMARY:

This protocol demonstrates how to use the plant host to detect salivary proteins of leafhopper and plant viral proteins released by leafhopper vectors.

ABSTRACT:

Insect vectors horizontally transmit many plant viruses of agricultural importance. More than one-half of plant viruses are transmitted by hemipteran insects that have piercing-sucking mouthparts. During viral transmission, the insect saliva bridges the virus-vector-host because the saliva vectors viruses, and the insect proteins, trigger or suppress the immune response of plants from insects into plant hosts. The identification and functional analyses of salivary proteins are becoming a new area of focus in the research field of arbovirus-host interactions. This protocol provides a system to detect proteins in the saliva of leafhoppers using the plant host. The leafhopper vector *Nephotettix cincticeps* infected with rice dwarf virus (RDV) serves as an example. The vitellogenin and major outer capsid protein P8 of RDV vectored by the saliva of *N. cincticeps* can be detected simultaneously in the rice plant that *N. cincticeps* feeds on. This method is applicable for testing the salivary proteins that are transiently retained in the plant host after insect feeding. It is believed that this system of detection will benefit the study of hemipteran-virus-plant or hemipteran-plant interactions.

INTRODUCTION:

The vector-host transmission mode of arboviruses, a fundamental problem, is at the frontier of biological science. Many plant viruses of agricultural importance are horizontally transmitted by insect vectors[1]. More than one-half of plant viruses are vectored by hemipteran insects, including aphids, whiteflies, leafhoppers, planthoppers, and thrips. These insects have distinct features that enable them to efficiently transmit plant viruses[1]. They possess piercing-sucking mouthparts and feed on the sap from phloem and xylem, and secrete their saliva[1–4]. With the development

and improvement of techniques, the identification and functional analyses of salivary components are becoming a new focus of intensive research. The known salivary proteins in saliva include numerous enzymes, such as pectinesterase, cellulase, peroxidase, alkaline phosphatase, polyphenol oxidase, and sucrase, among others[5–13]. The proteins in saliva also include elicitors that trigger the host defense response, thereby altering the performance of insects, and effectors that suppress the host defense, which enhances insect fitness and components that induce host pathological responses[14–17]. Therefore, saliva proteins are vital materials for communication between insects and hosts. During the transmission of viruses, the saliva secreted by the salivary glands of piercing-sucking viruliferous insects also contains viral proteins. Viral components utilize the flow of saliva to release them from the insect to the plant host. Therefore, the insect saliva bridges the virus-vector-host tritrophic interaction. Investigating the biological function of saliva proteins secreted by viruliferous insects helps to understand the relationship of virus-vector-host.

For animal viruses, it is reported that the saliva of mosquitoes mediates the transmission and pathogenicity of West Nile virus (WNV) and Dengue virus (DENV). The saliva protein AaSG34 promotes dengue-2 virus replication and transmission, while the saliva protein AaVA-1 promotes DENV and Zika virus (ZIKV) transmission by activating autophagy[18,19]. The saliva protein D7 of mosquitoes can inhibit DENV infection *in vitro* and *in vivo via* direct interaction with the DENV virions and recombinant DENV envelope protein[20]. In plant viruses, the begomovirus tomato yellow leaf curl virus (TYLCV) induces the whitefly salivary protein Bsp9, which suppresses the WRKY33-mediated immunity of plant host, to increase the preference and performance of whiteflies, eventually increasing the transmission of viruses[21]. Because studies of the role that insect salivary proteins play in plant hosts have lagged behind those of animal hosts, a stable and reliable system to detect the salivary proteins in plant hosts is urgently required.

The plant virus known as rice dwarf virus (RDV) is transmitted by the leafhopper *Nephotettix cincticeps* (Hemiptera: Cicadellidae) with high efficiency and in a persistently propagative manner[22,23]. RDV was first reported to be transmitted by an insect vector and causes a severe disease of rice in Asia[24,25]. The virion is icosahedral and double-layered spherical, and the outer layer contains the P8 outer capsid protein[22]. The circulative transmission period of RDV in *N. cincticeps* is 14 days[26–30]. When the RDV arrives at salivary glands, virions are released into saliva-stored cavities in the salivary glands *via* an exocytosis-like mechanism[23]. The vitellogenin (Vg) is the yolk protein precursor essential for oocyte development in female insects[31–33]. Most insect species have at least one Vg transcript of 6–7 kb, which encodes a precursor protein of approximately 220 kDa. The protein precursors of Vg can usually be cleaved into large (140 to 190 kDa) and small (<50 kDa) fragments before entering the ovary[18,19]. Previous proteomic analysis revealed the presence of the peptides derived from Vg in the secreted saliva of the leafhopper *Recilia dorsalis*, although their function is unknown (unpublished data). It is newly reported that Vg, which is orally secreted from planthoppers, functions as an effector to damage the defenses of plants[34]. It is unknown whether the Vg of *N. cincticeps* could also be released to the plant host with salivary flow, and then could play a role in the plant to interfere with plant defenses. To address whether *N. cincticeps* exploits salivary proteins, such as Vg, to inhibit or activate plant defenses, the first step is identifying proteins released to the plant during feeding.

Understanding the method to identify the salivary proteins present in the plant is potentially essential to explain the function of saliva proteins and the interactions between Hemiptera and plants.

In the protocol presented here, *N. cincticeps* is used as an example to provide a method to examine the presence of salivary proteins in the plant host introduced through insect feeding. The protocol primarily details the collection and detection of salivary proteins and is helpful for further investigation on most hemipterans.

PROTOCOL:

The non-viruliferous adult leafhoppers were propagated in the Vector-borne Virus Research Center in Fujian Agriculture and Forestry University, China.

1. Nonviruliferous insect rearing

1.1. Rear the adults on rice seedlings in a cube cage that is 40 cm x 35 cm x 20 cm (length x width x height). Keep one side of the cage covered with an insect-proof net for ventilation.

1.1.1. Keep the cages with leafhoppers in an incubator that contains an in-built humidity controller at 26 °C with a relative humidity of 60%–75% under a photoperiod of 16 h light and 8 h dark.

1.2. Use an aspirator to gently transfer all the adults from their cage into a new cage that contains fresh rice seedlings each week.

1.2.1. Let more than 200 adults mate and lay eggs in the rice.

1.2.2. Retain the old rice seedlings for the nymphs to emerge. Rear these new nonviruliferous nymphs to the 2-instar stage.

2. Virus acquisition and the collection of viruliferous insects

2.1. Carefully transfer the 2-instar nonviruliferous nymphs to a glass culture tube (2.5 cm in diameter by 15 cm high) for 1–2 h for starvation using the aspirator.

2.1.1. Release the nymphs to a cage that contains an RDV-infected rice plant grown in a pot.

2.1.2. Allow the nymphs to feed on the infected rice plant for 2 days.

NOTE: Carefully water the rice plant and avoid washing away the nymphs. The 2-instar nymphs are approximately 1.6–2 mm long.

2.2. Carefully transfer these nymphs to a new cage that contains fresh virus-free rice seedlings with a relative humidity of 60%–75% under a photoperiod of 16 h light and 8 h dark. Allow the

nymphs to feed on the infected rice plant for 12 days to complete the circulative transmission period of RDV.

3. Collection of salivary proteins using a feeding cage

3.1. Prepare five small pipe-like feeding cages (2.5 cm in diameter by 4 cm high) in which one end is covered with insect-proof netting.

3.2. Confine 15–20 leafhoppers in each feeding cage, and then cover the other end of the cage with a thin foam mat.

3.2.1. Fix one rice seedling (5–6 cm high) between the end of cage and a foam mat with tapes. Ensure that the leafhoppers in the feeding cage can feed on the rice seedlings exposed to the interior of the cage.

3.3. Immerse the seedling roots in water so that the rice plant will remain alive. Allow the leafhoppers to feed on them for 2 days.

3.4. Remove the leafhoppers from their feeding cages and collect the rice seedlings on which they fed. Cut the parts of seedlings outside of the cage and recover the feeding regions of seedlings.

NOTE: This sample can be stored at -80 °C for 3 months at the most, if it is not instantly used for detection.

4. Reagent preparation

4.1. Dissolve 15.1 g of Tris-base, 94 g of glycine, and 5 g of SDS in 1 L of sterile water to prepare 5xTris-glycine buffer. Dilute 200 mL of 5xTris-glycine buffer with 800 mL of sterile water to prepare 1xTris-glycine buffer (see **Table 1** for buffer composition).

4.2. Dissolve 80 g of NaCl, 30 g of Tris-base, and 2 g of KCl in 1 L of sterile water to prepare 10xTris-buffered saline (TBS) buffer. Autoclave the solution at 121 °C for 15 min.

4.3. Dissolve 8 g of SDS, 4 mL of ß-mercaptoethanol, 0.02 g of bromophenol blue, and 40 mL of glycerol in 40 mL of 0.1 M Tris-HCl (pH 6.8) to prepare 4x protein sample buffer.

4.4. Mix 800 mL Tris-glycine buffer with 200 mL methanol to prepare the transfer buffer.

4.5. Add 100 mL 10xTBS solution and 3 mL Tween 20 to 900 mL sterile water to prepare the TBS buffer with Tween 20 (TBST) solution.

5. Western blotting to detect the saliva and viral proteins

5.1. Grind 0.1 g of the rice samples with liquid nitrogen until the tissue becomes a powder. Add 200 µL of 4x protein sample buffer to the sample and boil it for 10 min. Centrifuge the samples at 12,000 x *g* for 10 min at room temperature.

5.1.1. Remove the supernatant and place it in a new vial. Load 10 µL of the sample into an SDS-PAGE gel, and run it in Tris-glycine buffer at 150 V for 45–60 min.

NOTE: The residue after centrifugation can be discarded.

5.2. Put a 0.45 µm nitrocellulose membrane and other sandwich supplies in the Transfer buffer for 30 min.

NOTE: This step can be done before the gel has completed its run.

5.3. Sandwich the gel and transfer it for 90–120 min at 100 V in the Transfer buffer.

5.4. Take the membrane and place it in 7% non-fat dry milk blocking solution in TBST solution for 20 min. Add the specific antibody against RDV P8 or Vg to a 7% solution of non-fat dry milk in TBST. Incubate with the antibody for staining the membrane for 2 h or overnight.

5.5. Wash the membrane with TBST solution three times, with 5 min washing each time.

5.6. Add the goat anti-rabbit IgG as a secondary antibody to 7% non-fat dry milk with TBST. Incubate with the antibody for 60–90 min at room temperature.

5.7. Wash the membrane with TBST solution three times for 5 min each time.

5.8. Use the ECL Western kit for the chemiluminescent method. Mix Detection Reagents 1 and 2 in the kit at a ratio of 1:1 in a tube. Put the mixed reagent onto the membrane and incubate the blot for 5 min.

5.9. Drain the excess reagent and take a colorimetric picture of the chemiluminescent picture. Combine them to see the ladder with protein bands.

REPRESENTATIVE RESULTS:

Figure 1 illustrates all of the steps in this protocol: insect rearing, virus acquisition, the collection of salivary proteins *via* rice feeding, and the western blot. The western blots results showed that specific and expected bands of approximately 220 kDa were observed in the samples of feeding rice and salivary glands of insects on the membrane incubated with antibodies against Vg. In contrast, no band was observed in the non-feeding rice sample. The result in **Figure 2** indicates that Vg was released to the plant host as a salivary protein. On the membrane incubated with antibodies against RDV P8, a specific and expected band of approximately 46 kDa was also observed in the samples from rice that had been subjected to feeding and the bodies of viruliferous insect bodies. In contrast, no band was observed in the non-feeding rice sample and

the nonviruliferous leafhopper bodies, as shown in **Figure 3**. This result proved that the viral proteins could also be detected in the feeding plants.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the steps in the detection of salivary proteins. The steps include insect rearing, virus acquisition, salivary proteins collection *via* plant feeding, and western blotting.

Figure 2: Western blot assay of Vg in plant and insect samples. Lane 1, non-feeding plants; Lane 2, viruliferous leafhopper feeding plants; Lane 3, viruliferous leafhopper salivary glands; Lane M, marker.

Figure 3: Western blot assay of P8 in plant and insect samples. Lane 1, non-feeding plants; Lane 2, viruliferous leafhopper feeding plants; Lane 3, viruliferous leafhopper bodies; Lane 4, nonviruliferous leafhopper bodies; M, marker.

Table 1: Buffers, solutions, and reagents used in the study. The composition of the buffers and the solutions, along with their usage, are listed.

DISCUSSION:

The saliva directly secreted by the salivary glands of the piercing-sucking insects plays a pivotal role because it predigests and detoxifies the host tissues and vectors' cross-kingdom biological factors into the hosts[1,3,4]. The cross-kingdom biological factors, including elicitors, effectors, and small RNA, are critical for insect-host communication[14–16]. Therefore, uncovering more varieties and functions of salivary components will promote understanding the relationship between insects and hosts. Here, a system of detection for salivary proteins in the plant host was provided, which will enable further investigation on the function of salivary protein in plant hosts.

This protocol provides techniques to detect the salivary proteins of leafhoppers with piercing-sucking mouthparts by collecting the feeding plants. Some remarkable points should be noted to obtain the best and reliable results. (1) The viral loading in the infected rice plants is critical. The viral titers in rice plants directly affect the acquisition of insects. When the insect colony is highly viruliferous, the probability of collecting viral proteins in saliva *in vitro* will be increased. Therefore, the viral proteins released from the saliva to the plant host will be much easier to detect. Choosing infected plants that display significant symptoms is recommended to serve as the source for viruses. (2) Detection timing. It is believed that some of the salivary proteins are transient in the plant host because they are subjected to degradation by the plant host or diluted in the plant. Instant detection of the feeding plants after the 2-day feeding is recommended. It is also hypothesized that a longer retention time would be better for some specific salivary proteins in the plant. Therefore, the detection timing of some salivary proteins could be determined in further studies. (3) Ensure that there are enough replicates. The number of insects that survive will decrease because the confined insects in the feeding cage have limited activity and ability to feed. Using enough replicates will help to enrich the salivary proteins. Three to five replicates are typically enough. If there is only one replicate, it would be better to confine 15–20 leafhoppers

to feed on one rice seedling.

These representative results showed the presence of viral protein P8 in the feeding plant of viruliferous leafhoppers. It was revealed that the virus mixed with the flow of saliva is released from the salivary glands. It was then released from the insect to the plant host, ultimately finishing the viral horizontal transmission. However, it is still unknown whether Vg plays the role of elicitor or effector in the process of insect feeding and whether or not it triggers or suppresses the immune response of the plant host. Previously, the saliva of more than 10,000 *R. dorsalis* was collected *via* membrane feeding and analyzed using LC-MS/MS (unpublished data). The presence of Vg in the saliva was verified, although its function is unknown. Here, it has been proven that Vg also exists in the saliva of *N. cincticeps* and is even released to the plant. Combined with the studies on planthoppers[16], it is presumed that the presence of Vg in hemipteran saliva is universal. A new finding reports that the Vg of planthopper saliva is an effector to damage the plant defense system[34]. More studies are required to address whether the Vg in most hemipteran saliva functions as an effector. It is believed that this protocol provides a stable and reliable methodology to examine the presence of salivary proteins secreted by leafhoppers in plants. This protocol is expected to be applicable for the detection of salivary proteins of most hemipterans.

ACKNOWLEDGMENTS:
This work was supported by grants from the National Natural Science Foundation of China (31772124 and 31972239) and Fujian Agriculture and Forestry University (Grant KSYLX014).

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

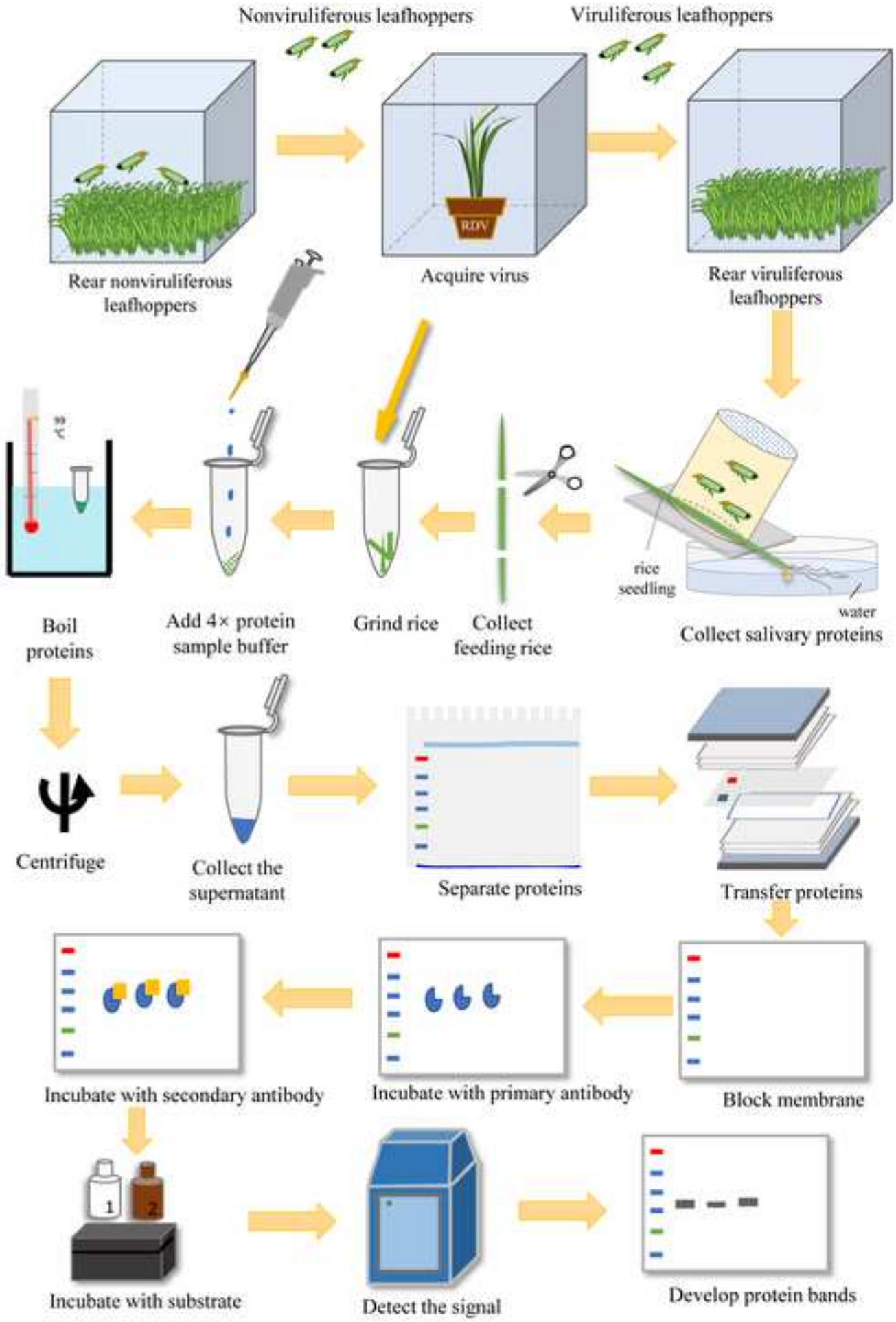

Figure 2

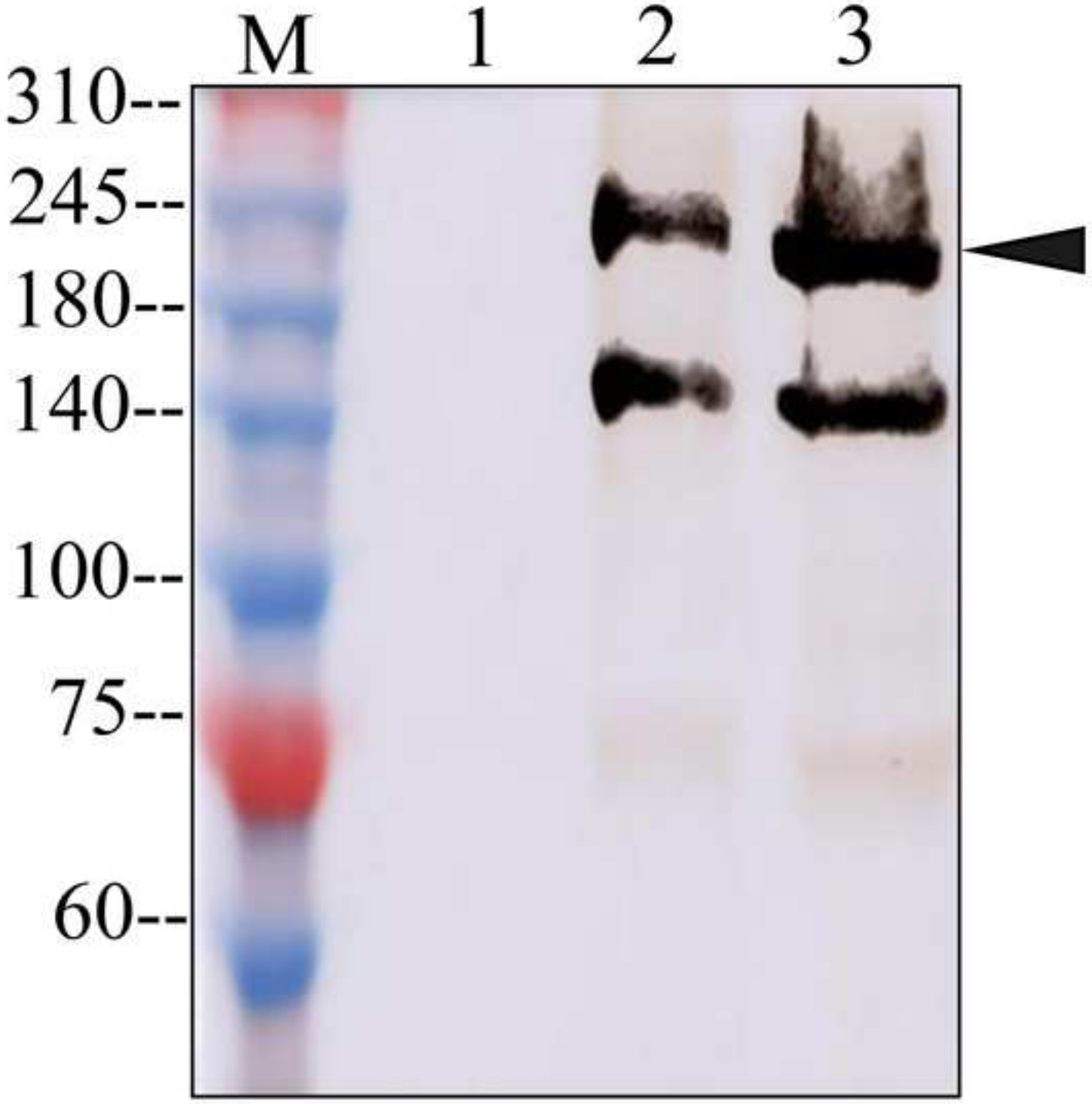

Figure 3

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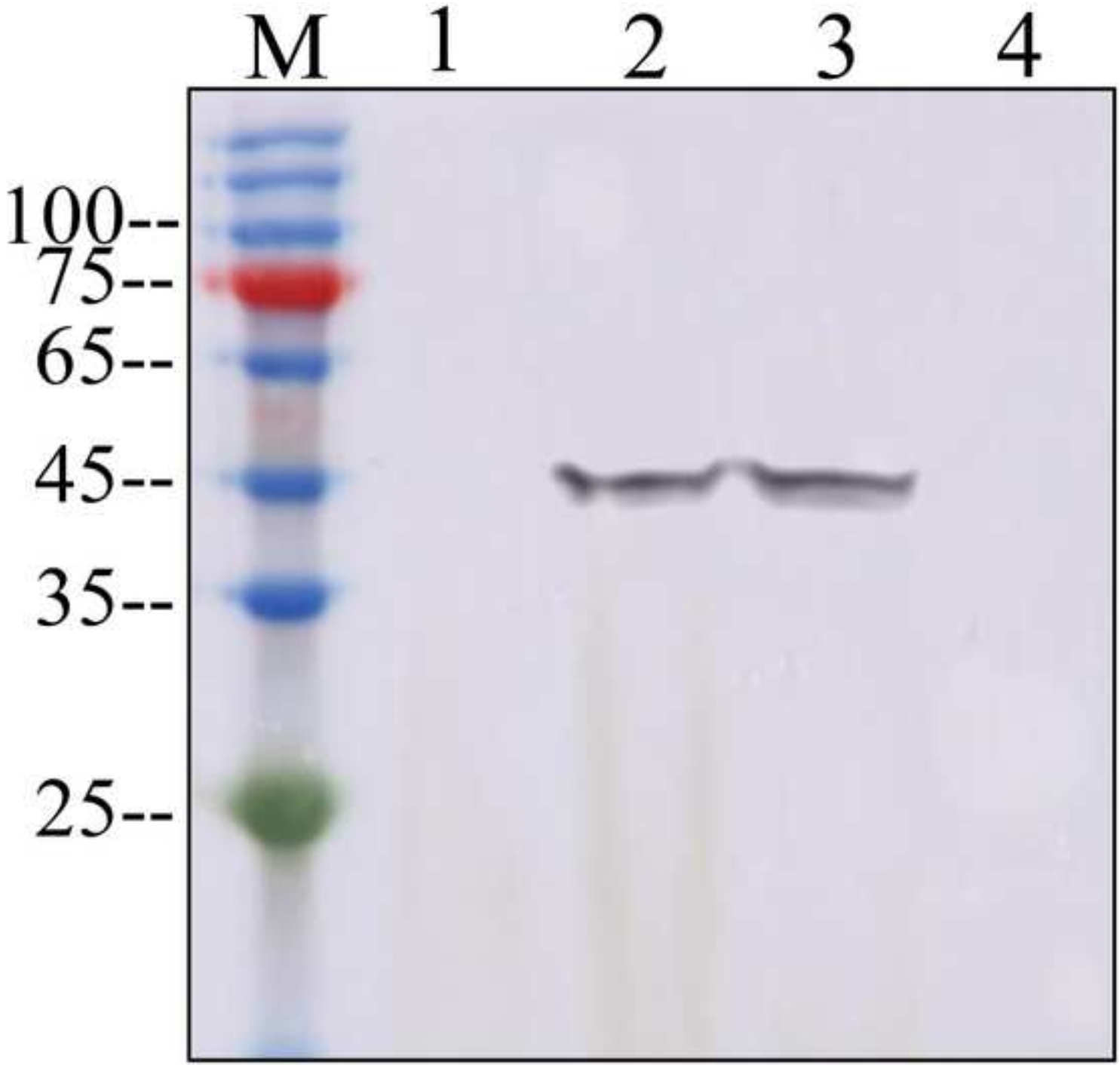

Table 1

Buffer	Composition
5x Tris-glycine buffer	15.1 g Tris base 94 g glycine 5 g SDS in 1 L sterile water
1x Tris-glycine buffer	200 mL of 5x Tris-glycine buffer 800 mL sterile water
10x Tris-buffered saline (TBS) buffer	80 g NaCl 30 g Tris base 2 g KCl in 1 L sterile water
TBS with Tween 20 (TBST) solution	100 mL 10x TBS solution 3 mL Tween 20 900 mL sterile water
4x protein sample buffer	8 g SDS 4 mL β-mercaptoethanol 0.02 g bromophenol blue 40 mL glycerol in 40 mL 0.1 M Tris-HCl (pH 6.8)
Transfer buffer	800 mL Tris-glycine buffer 200 mL methanol

	Comments/Description
Stock solution	
Work solution, for SDS-PAGE	
Stock solution	
Work solution	
For protein extraction	
For protein transfer	

Aug 30, 2021
The Editorial Office, *JoVE*

Re: #JoVE63020R1 (A protocol to detect virus and salivary proteins of a leafhopper vector in the plant host)

Dear Review Editor,

Thank you for your edit of our manuscript entitled “A protocol to detect virus and salivary proteins of a leafhopper vector in the plant host (#JoVE63020R1)”. Those edits are all valuable and very helpful. We have carefully revised the manuscript. We hope the current version is suitable for publication in *JoVE*.

Yours sincerely,

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Editorial comments:

Changes to be made by the Author(s):

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

Response: The manuscript was edited by a professional.

2. Please revise the following lines to avoid previously published work: 14-16, 32-34,73-75, 77-80.

Response: They were revised.

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

Response: The text was revised.

4. Please ensure that abbreviations are defined at first usage.

Response: They were checked and defined at their first usage.

5. 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. For example, please remove the commercial term "Pierce" from Line 150. All commercial products should be sufficiently referenced in the Table of Materials (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material.

Response: This has been revised, and the instruments were added to the Table of Materials.

6. 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 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: This was checked and revised. The "how" questions were answered as thoroughly as possible.

7. Please add more details to your protocol steps:

Step 1.1: How can you ensure that the rice field is virus-free? Also, please clarify whether the incubator has an in-built humidity controller?

Response: These questions were answered.

Step 1.2: What is the indication of the 2-instar stage?

Response: This information was provided.

Step 3.2: How are rice seedlings fixed?

Response: This information was provided.

Step 3.3: How long can the sample be stored?

Response: This information was provided.

Step 5.1: Please mention the centrifugation temperature. The residue is discarded, or what happens with that?

Response: This information was provided.

Step 5.6: This step is performed at room temperature?

Response: This information for this step was provided.

8. Please include the compositions of all the buffers used for the study, along with the pH for each. You may provide all these compositions in a separate Table. Please ensure that the Table of Materials has the details of all the essential supplies, reagents, and equipment.

Response: The compositions of all the buffers were added to the Table of Materials.

9. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

Response: The text was revised.

10. 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

Response: The text was revised.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors investigated the possible presence of virus and protein of saliva, both vectored by plant feeding insects, in rice. The topic is potentially important to disentangle epidemiology of vector-borne plant virus. However, the authors did not explain properly how this could be useful for future research. I feel that there are many gaps in the manuscript, among the others the most important are:

-Introduction: poorly addressed, lacking of links between topics. No clear statements for future applications

-Material and Methods: some details to reproduce the protocol are missing

- Result & Discussion: providing only evidence on detection of Vg and virus in rice, I think that the Discussion does not entirely fit the paper.

I strongly suggest the authors consider extensive improvement.

Response: The text was revised.

Moreover, I believe that the manuscript needs extensive revision for English language and grammar, including punctuation. As it is, the text appears confusing and several sentences mislead the readers.

Response: The manuscript was edited by a professional.

Major Concerns:

Title: please consider changing the title. It is a little bit confusing and vague. As it is, the paper should be titled: "A protocol to detect ….. ". I understand that it is a methodological paper, then it should be clear in the title.

Response: The title was revised.

Line 40: is it "behaviors" or fitness?

Response: This line was revised.

Line 38-42: this paragraph is not clear. The authors mentioned several mechanisms and interactions without any clear link among them, and it is not clear where the narrative is leading the reader. In particular, I do not believe that the last sentence is a mere consequence of the previous one. As well, is not just the saliva that bridges insect-vector-plant interaction. Please reward both of them.

Line 57: there is no need to repeat this sentence here. I suggest considering the previous comment and edit this part as well. I would focus more on the simple role of saliva in the tritrophic interaction, saliva is actually a bridge only for virus and other molecules to pass between insect and plant. But does not have anything to do with the insect-virus interaction or insect-plant interaction, where mainly other processes and mechanisms are involved.

Response: There is strong evidence that saliva plays an important role in insect-virus interactions or insect-plant interactions. Saliva vectors viruses and molecules from insects to plants. Since the salivary component involves elicitors and effectors, which play roles in the activation or inhibition of several plant defense responses against insects, the saliva is crucial for the insect-plant interaction (Hogenhout and Bos, 2011; Tomkins et al., 2018; Huang et al., 2018; Ji et al., 2021). In addition, it has been reported that infection with viruses can change the salivary components, including salivary proteins and sRNA. These changes affect the defenses of plant against pathogens or insects, or they affect the fitness of insect for feeding, ultimately facilitating passage of the virus from insects to plants

(Comway et al., 2016; Wang et al., 2019). Taken together, saliva plays a vital role in insect-virus interactions and insect-plant interactions.

Line 74-75: this sentence can be removed; it is not central for the narrative.

Response: It was removed.

Line 82: the authors should mention here, if know, why Vg was found in saliva (function ...), if it is not known they should mention as well.

Response: It is unknown why Vg was in the saliva of leafhopper *Recilia dorsalis*. However, it has been recently reported that Vg in the saliva of a planthopper acts as an effector to damage the defenses of plants. This narrative was added on lines 72-73.

Line 82-83: this sentence looks like a hypothesis. I suggest rewording the sentence to make a real strong hypothesis, by including why the authors had reasons to believe that Vg could be found in N. cincticep's saliva and plant host, and why this would be potentially important. Why the authors are providing this protocol ?

Response: This sentence was revised, and the reasons were added on lines 73-75.

Line 84-90: this is too much MAterial and MEthods and Results anticipation. I would suggest drastically reduce and improve the hypothesis of the present study, as suggested above.

Response: The text was revised.

Line 94: which field? It is virus-free area? where it is. Please specify

Response: This line was revised.

Line 94-100: did you collected adults or nymphs? Did you let the adults mating and lay

eggs in the cage?

Response: The additional information was provided for these lines.

Line 106: I would delete this note which pertain to the ability and training of the person who carried out the experiment.

Response: The authors convey their thanks and deleted the note.

Line 157-164: this belongs to M&M

Response: This section was revised.

Line 187-202: because the authors do not have final evidence from this study (and actually it was only a detection) for the Vg and saliva in N. cincticeps playing the roles discussed here for mosquito, I think that this part has to be moved in the Introduction. In fact, there are not results that can be discussed with these previous experiences and findings. Overall, I think that the paper is useful to provide a reliable protocol for protein and virus detection. However, there is no discussion with respect to the previous findings they reported in the Introduction on Recilia dorsalis. I think this point is crucial for the present journal and it should be further discussed in this section.

Response: This part was added to the Introduction. In addition, previous information on the vg of *Recilia dorsali* was added and discussed.

Reviewer #2:

This protocol describes how to detect virus and salivary porteins of hemipteran vectors in plant host. This protocol will be usefurl for further studies about hemipteran-virus-plant or hemipteran-plant interactions.

Response: The authors convey their thanks.

I have a couple of minor comments:

1. This protocol used the leafhopper vector Nephotettix cincticeps infected with rice dwarf virus (RDV) as an example. But the title is hemipteran vector. Could this protocol be suitable for other hemipteran vectors? Please re-consider the title or discuss the availability for other Response: The title was revised.

2. In the discussion, some remarkable points for best and reliable results were noted. "Enough replicates" should be adopted. It is better to give the detail recommendations about how many replicates is enough. For one replicate, how many insects and how many rice seedlings were used?

Response: This section was expanded and discussed in more detail.