AUTHOR REPLY

Reply to "Conclusive Evidence of Replication of a Plant Virus in Honeybees Is Lacking"

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We appreciate Miller et al. (1) for taking the time to offer comments related to our paper "Systemic Spread and Propagation of a Plant-Pathogenic Virus in European Honeybees, *Apis mellifera*" (2). We provide the following responses for clarification.

The study on the pathogenesis of virus infections in honeybees has been hampered by the lack of a robust cell culture system for viral replication. The detection and quantification of negative-strand RNA intermediates offer an excellent alternative for the demonstration of virus replication and pathogenesis in infected hosts. However, there have been concerns regarding strand specificity of the method due to false priming events during reverse transcription, which, as pointed out by Miller et al., could result in nonspecific strand amplification. We remained on high alert regarding the limitations of this method during our study and validated each step of the assay for strand-specific amplification of tobacco ringspot virus (TRSV). According to our pilot studies, the major problem associated with amplification of nontargeted RNA strand (i.e., positive-stranded RNA) using a tagged reverse transcription (RT)-PCR assay was due to carryover of tagged forward primer from the RT to the subsequent PCR amplification. The carryover of tagged forward primer and reverse primer could initiate the amplification of cDNA copies of not only positive polarity but also negative polarity that was due to false priming at the RT step, causing the loss of the strand specificity of the assay. Alternatively, if the residue-tagged forward primers from RT were removed effectively, the negative-strand RNA of the virus could be specifically amplified with tagged primer and reverse primer even if done in the possible presence of cDNA copies of both positive and negative polarity such as those used with the same PCR amplification mechanism for target template mediated by specific primers. For this reason, we improved the assay by employing double cDNA purification. The cDNA generated by tagged forward primer for negative-strand RNA was first purified by using a MinElute PCR purification kit (Qiagen) to allow for the effective collection of cDNA of 70 bp to 4 kb in size and then was further purified using a MinElute reaction clean kit (Qiagen) to remove short fragments of oligonucleotides and residue of enzymatic reagents. Our studies proved that the strand specificity could be ensured by the combination of tagged RT-PCR and cDNA purification and relieved our concerns about the amplification of the positive-strand RNA template. While detection and quantification of both strands of RNA of TRSV were performed in our studies (the proportion of negative- to positive-strand RNA was about 1:4), the specific focus of this particular paper was the spread and propagation of TRSV in different tissues of honeybees; the results about validation of the specificity of the assay for detection of both negative- and positive-strand RNA of TRSV were not included.

Regarding the suggestion of using RNA from purified TRSV virions as a template for RT-PCR to address the possibility of encapsulation/packaging of replicative RNA intermediates, it is not clear to us how this test would help rule out the possibility of nonspecific amplification of positive-strand RNA from different bee tissues. According to our experience, false priming during cDNA synthesis is almost inevitable regardless of the inclusion of forward primers, reverse primers, or both primers or even without primers in the RT. However, the use of tagged forward primer in the RT would lead to a high abundance of cDNA synthesis from negative-strand RNA relative to the cDNA synthesis from positive-strand RNA due to false priming events. The specificity of our method was confirmed by performing PCR amplification using opposite-sense RNA as a template (amplifying cDNA synthesized by tagged forward primer in the presence of tagged primer and forward primer and vice versa). After confirmation of negative amplification using opposite-sense RNA as a template, we moved forward and employed this validated method to answer our question of whether TRSV could cause systemic infection and replicate in honeybees.

While our report provided evidence only of the presence of TRSV in *Varroa destructor*, it would be beneficial to know if parasitic mites could support replication of TRSV. The suggestion of monitoring the changes in TRSV levels over time by inoculating TRSV-free honeybees with TRSV to test infectivity is a good one and will be included in our ongoing studies.

REFERENCES

1. **Miller WA, Carrillo-Tripp J, Bonning BC, Dolezal AG, Toth AL.** 2014. Conclusive evidence of replication of a plant virus in honeybees is lacking. mBio 5(3):e00985-14. http://dx.doi.org/10.1128/mBio.00985-14.
2. **Lian JL, Cornman RS, Evans JD, Pettis JS, Zhao Y, Murphy C, Peng WJ, Wu J, Hamilton M, Boncristiani HF, Jr, Zhou L, Hammond J, Chen YP.**

Published 27 May 2014

Citation Li JL, Cornman SR, Evans JD, Pettis JS, Zhao Y, Murphy C, Peng WJ, Wu J, Hamilton M, Boncristiani HF, Jr., Zhou L, Hammond J, Chen YP. 2014. Reply to "Conclusive Evidence of Replication of a Plant Virus in Honeybees Is Lacking." mBio 5(3): e01250-14. doi:10.1128/mBio.01250-14.

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