In vitro and in vivo host range of Anopheles gambiae densovirus (AgDNV)

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AgDNV is a powerful gene transduction tool and potential biological control agent for Anopheles mosquitoes. Using a GFP reporter virus system, we investigated AgDNV host range specificity in four arthropod cell lines (derived from An. gambiae, Aedes albopictus and Drosophila melanogaster) and six mosquito species from 3 genera (An. gambiae, An. arabiensis, An. stephensi, Ae. albopictus, Ae. aegypti and Culex tarsalis). In vitro, efficient viral invasion, replication and GFP expression was only observed in MOS55 An. gambiae cells. In vivo, high levels of GFP were observed in An. gambiae mosquitoes. Intermediate levels of GFP were observed in the closely related species An. arabiensis. Low levels of GFP were observed in An. stephensi, Ae. albopictus, Ae. aegypti and Cx. tarsalis. These results suggest that AgDNV is a specific gene transduction tool for members of the An. gambiae species complex, and could be potentially developed into a biocontrol agent with minimal off-target effects.

Densoviruses (DNVs) are non-enveloped single-stranded DNA viruses in the family Paroviridae. DNVs are broadly distributed in invertebrates and are often pathogenic to their hosts¹⁻⁶. Many DNVs have been isolated from various laboratory and field mosquitoes and cell lines⁷⁻⁹. The Anopheles gambiae densovirus (AgDNV) is highly infectious and capable of transducing exogenous genes in An. gambiae, the major human malaria vector in Sub-Saharan Africa⁹,¹⁰. Unlike most mosquito densoviruses, AgDNV exhibits negligible pathology in An. gambiae¹¹. These features make AgDNV an attractive candidate for paratransgenesis, an approach that renders insects refractory to pathogens by using transgenic microbes¹²⁻¹⁴. The use of paratransgenesis in the field needs to be considered carefully and unwanted side effects such as off-target infections need to be investigated. Understanding basic aspects of viral ecology such as host range is crucial to evaluate feasibility of viral paratransgenesis in the field.

Densovirus host range has been studied with the Aedes aegypti densovirus (AeDNV) and Aedes albopictus densovirus (AalDNV). AeDNV and AalDNV are infectious to Aedes, Culex and Culiseta mosquitoes, but are not infectious to other insects or vertebrates¹⁵⁻¹⁶. Among mosquitoes, Ae. aegypti and Ae. albopictus show relatively high susceptibility to multiple mosquito densoviruses¹⁷⁻¹⁹. Ward et al. used a recombinant AeDNV expressing green fluorescent protein (GFP), and demonstrated that AeDNV can fully disseminate in Ae. aegypti but only infects the anal papillae or bristle cells and does not disseminate in An. gambiae²⁰. Previous work by our lab led to the isolation of AgDNV and the development of GFP-expressing recombinant virus, which is capable of efficiently infecting and disseminating in An. gambiae²¹. In this study, we used this system to investigate the host range of AgDNV in multiple invertebrate cell lines and mosquito species.

Results
In vitro AgDNV host specificity. MOS55 (An. gambiae), Sua5B (An. gambiae), C6/36 (Ae. albopictus) and S2 (Drosophila melanogaster) cell lines were infected with 1 × 10⁹ virions of recombinant
GFP-expressing AgDNV (vUTRAcGFP). Three days post-infection, GFP expression levels were examined using fluorescence microscopy, flow cytometry and quantitative PCR (qPCR). MOS55 cells showed the highest GFP fluorescence (Fig. 1A,B). Low levels of GFP were observed in C6/36 cells and no fluorescence was observed in Sua5B or S2 cells (Fig. 1A,B). Using qPCR, levels of viral DNA copies of vUTRAcGFP matched results obtained by flow cytometry (Fig. 1C).

**In vivo AgDNV host specificity.** We next investigated viral host range among mosquito species in vivo using *An. gambiae, An. stephensi, An. arabiensis, Ae. aegypti, Ae. albopictus* and *Culex tarsalis*. 40–50 adult mosquitoes of each species were injected with $1 \times 10^7$ of vUTRAcGFP. At 7 days post injection, mosquitoes were visually examined for GFP expression using fluorescence microscopy. We defined a seven category scoring criteria (0–6) for the level of fluorescence expression in individual mosquitoes (Fig. 2). This scoring system allowed us to compare the viral infection levels semi-quantitatively and analyze the distribution of GFP expression level within each species. The known permissive mosquito, *An. gambiae*, exhibited scores ranging from 3 to 6 with an average score of 4.8 (Fig. 3A,G). The closely related species *An. arabiensis* exhibited scores ranging from 2 to 5 with an average score of 3.3 (Fig. 3C,G). In other mosquito species, the distributions were shifted and had statistically significantly lower ranges.
An. stephensi, the closest relative of An. gambiae and An. arabiensis examined in this study, had an average score of only 1.0 (Fig. 3B,G). Ae. aegypti, Ae. albopictus and Cx. tarsalis had average scores of 0.47, 1.2 and 0.40 respectively (Fig. 3D–G).

Discussion

Although AgDNV was originally isolated from An. gambiae Sua5B cells9, no fluorescence or viral DNA was detected after vUTRAcGFP infection of this cell line. However, Sua5B cells are permissive to viral replication of the naturally occurring virus present in the cell line or if transfected with a recombinant infectious clone plasmid9. These results suggest that Sua5B cells may have been originally infected with AgDNV from the original mosquito colony from which the cell line was established, however, during development of the cell line and/or over long-term serial passage the cells lost essential host factors (such as receptors) required for new infection. In contrast, the An. gambiae MOS55 cell line retains these factors and is permissive to infection. Comparison of these two cell lines may help identify the specific receptors required for AgDNV entry into cells.

The An. gambiae species complex consists of at least seven morphologically identical mosquito species, to which both An. gambiae and An. arabiensis belong19. We had initially hypothesized that AgDNV would in general infect Anopheline mosquitoes better than species belonging to other genera. However, this was not the case. While An. gambiae, and to a lesser extent An. arabiensis were susceptible to infection, the congeneric species An. stephensi was refractory to infection (Fig. 3B,G). An. stephensi is the major Asian vector of human malaria and is not part of the gambiae complex19. These observations suggest that AgDNV is specifically adapted to infect An. gambiae and closely related species.

We unexpectedly observed intermediate to high levels of GFP expression (scores of 3 to 5) in a low percentage of Ae. albopictus individuals (Fig. 3D), leading to a significantly higher mean infection score for Ae. albopictus compared to Ae. aegypti or Cx. tarsalis (P < 0.05) (Fig. 3D–G). These results complement our cell line data, where C6/36 cells (derived from Ae. albopictus) were also minimally permissive to viral infection. To date, there are no reports detailing the molecular mechanisms underlying host specificity of mosquito densoviruses. Clathrin-mediated endocytosis has been shown to be important for infection for mammalian and insect paroviruses such as canine parovirus (CPV) and Junonia coenia densovirus (JcDNV)20–22. The clathrin-mediated endocytosis pathway is likely used by mosquito densoviruses as well. Structural variation of the receptors and downstream molecules could determine the host specificity of AgDNV among mosquito species and remains to be investigated.

Figure 2. Representative images of vUTRAcGFP-infected mosquitoes for scoring GFP expression. Fluorescence levels were categorized into 7 categories (0–6) based on the indicated criteria. An. stephensi (scores, 0–2) and An. gambiae (scores, 3–6) are shown as representative examples.
Unlike other densoviruses that are highly pathogenic to their hosts, AgDNV has a negligible impact on the life span of *An. gambiae*\(^1\)–\(^6\),\(^11\). The highly infectious but non-pathogenic and specific nature of the interaction between *An. gambiae* and AgDNV suggests a history of co-evolution and host-specific adaptation in this system that is distinct from other studied mosquito densoviruses. Further experiments to elucidate the molecular mechanisms of this observed specificity will provide mechanistic insights into the evolution of host-specific pathogens, and will inform on the utility of using DNVs for targeted biocontrol of vector mosquitoes in the field.

Figure 3. Comparison of GFP infection scores in six mosquito species. (A) *An. gambiae*, (B) *An. stephensi*, (C) *An. arabiensis*, (D) *Ae. albopictus*, (E) *Ae. aegypti* and (F) *Cx. tarsalis*. (G) Mean infection score for each mosquito species. Data were analyzed by ANOVA with Bonferroni’s correction for multiple comparisons. Letters represent statistical significance (P < 0.05).
Methods

Transducing virus production. Virions of recombinant GFP-expressing AgDNV (vUTRAcGFP) were produced by co-transfection of MOS55 cells with the recombinant virus plasmid pUTRAcGFP and the wild type AgDNV helper plasmid pBAG5x as described9–10. Viral titer for these infection experiments was determined with qPCR using a standard curve as previously described11. Briefly, DNV samples were TURBO DNase (Ambion) treated to digest plasmid DNAs. Total DNA was extracted using DNeasy kits (Qiagen). qPCR was performed using the QuantiTect SYBR Green Kit (Qiagen) on a Rotor-Gene Q (Qiagen) with EGFP primers: 5′-TCA-AGA-TCC-GGC-ACA-ACA-TC 3′, 5′-TTC-TCG-TTG-GGG-TCT-CTG-CT 3′. A standard curve was created using a dilution series of pUTRAcGFP ranging from 10^3 to 10^9 copies.

In vitro AgDNV infection quantitation. MOS55 (An. gambiae), Sua5B (An. gambiae), C6/36 (Ae. albopictus) and S2 (Drosophila melanogaster) cell lines were cultured in Schneider's media with 10% fetal bovine serum. Cells were infected with 1 × 10^5 virions of recombinant GFP-expressing AgDNV (vUTRAcGFP)10. Viral DNA level in infected cells was determined by qPCR as described above. GFP mean fluorescence intensity (MFI) was determined using flow cytometry with FlowJo software. Statistical differences between treatments were determined using analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons.

In vivo AgDNV infection quantitation. Mosquitoes of each species (An. gambiae [Keeler strain], An. stephensi [Liston strain], An. arabiensis [Dongola strain], Ae. aegypti [Rock strain], Ae. albopictus [Houston strain] and Culex tarsalis [Yolo strain]) were held at 27°C and 80% relative humidity and were maintained on expired human blood or commercially obtained bovine blood using a membrane feeding system, and were allowed access to 10% sucrose solution ad libitum through a cotton wick. For each species, 3–5 day old females were anesthetized by chilling and injected with 1 × 10^5 vUTRAcGFP using a glass capillary needle. Injected mosquitoes were held at 27°C and 80% relative humidity with 10% sucrose. A 7 category scoring scale (Fig. 2) was used to visually quantify GFP expression using an Olympus BX40 epifluorescent microscope at 7 days post-injection. Statistical differences between treatments were determined using analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. An. gambiae injected with media were used as a negative control.

References

1. Carlson, J., Suchman, E. & Buchatsky, L. Densovirus for control and genetic manipulation of mosquitoes. Adv. Virus Res. 68, 368–2 (2006).
2. Hewson, I. et al. Densovirus associated with sea-star wasting disease and mass mortality. Proc. Natl. Acad. Sci. USA. 111, 17278–3 (2014).
3. Mutuel, D. et al. Pathogenesis of Junonia coenia densovirus in Spodoptera frugiperda: a route of infection that leads to hypoxia. Virology. 403, 137–4 (2010).
4. Lightner, D. V., Redman, R. M. & Bell, T. A. Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. J. Invertebr. Pathol. 42, 62–70 (1983).
5. Ledermann, J. P., Suchman, E. L., Black, W. C. 4th & Carlson, J. O. Infection and Pathogenicity of the Mosquito Densovirus AeDNV, HeDNV, and APeDNV in Aedes aegypti Mosquitoes (Diptera: Culicidae). J. Econ. Entomol. 97, 1828–35 (2004).
6. Cornet, M. A Parvo-like virus persistently infecting a C6/36 clone of Aedes albopictus mosquito cell line and pathogenic for Aedes aegypti larvae. Virus Res. 29, 99–114 (1993).
7. Ma, M. et al. Discovery of DNA viruses in wild-caught mosquitoes using small RNA high throughput sequencing. PLoS One 6, e24758 (2011).
8. Zhai, Y. G. et al. Isolation and characterization of the full coding sequence of a novel densovirus from the mosquito Culex pipiens pallens. J. Gen. Virol. 89, 195–9 (2008).
9. Ren, X., Hoiczky, E. & Rasgon, J. L. Viral paratransgenesis in the malaria vector Anopheles gambiae. PLoS Pathog. 4, e1000135 (2008).
10. Suzuki, Y., Niu, G., Hughes, G. L. & Rasgon, J. L. A viral over-expression system for the major malaria mosquito Anopheles gambiae Sci. Rep. 4, 5127 (2014).
11. Ren, X., Hughes, G. L., Niu, G., Suzuki, Y. & Rasgon, J. L. Anopheles gambiae densovirus (AgDNV) has negligible effects on adult survival and transcriptome of its mosquito host. PLoS 2, e584 (2014).
12. Ren, X. & Rasgon, J. L. Potential for the Anopheles gambiae densovirus to act as an “evolution proof” biopesticide. J. Virol. 84, 7726–9 (2010).
13. Fang, W. et al. Development of transgenic fungi that kill human malaria parasites in mosquitoes. Science 331, 1074–7 (2011).
14. Wang, S. et al. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. Proc. Natl. Acad. Sci. USA 109, 12734–9 (2012).
15. O'Neill, S. L. et al. Insect densovirus may be widespread in mosquito cell lines. J. Invertebr. Pathol. 89, 62–72 (1999).
16. Kittayapong, P., Baisley, K. & O'Neill, S. A mosquito densovirus infecting Aedes aegypti and Aedes albopictus from Thailand. Am. J. Trop. Med. Hyg. 61, 612–9 (1999).
17. Afanasiev, B. N., Ward, T. W., Beatty, B. J. & Carlson, J. O. Transduction of Aedes aegypti mosquitoes with vectors derived from Anopheles densovirus. Virology 257, 62–72 (1999).
18. Ward, T. W. et al. Aedes aegypti transducing densovirus pathogenesis and expression in Aedes aegypti and Anopheles gambiae larvae. Insect Mol. Biol. 10, 397–405 (2001).
19. Neafsey, D. E. et al. Mosquito genomics. Highly evolvable malaria vectors: the genomes of 16 Anopheles mosquitoes. Science 347, 125822 (2015).
20. Vendevelle, A. et al. Densovirus infectious pathway requires clathrin-mediated endocytosis followed by trafficking to the nucleus. J. Virol. 83, 6678–89 (2009).
21. Farbizans, C. E., Chiorini, J. A. & Parrish, C. R. The paravirus capsid odyssey: from the cell surface to the nucleus. Trends Microbiol. 16, 208–14 (2008).
22. Parker, J. S. L. & Parrish, C. R. Cellular uptake and infection by Canine Parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. *J. Virol.* **74**, 1919–30 (2000).

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**Author Contributions**
Y.S. designed and performed the experiments, analyzed the data and contributed to drafting the manuscript. T.K.B. performed the experiments and contributed to drafting the manuscript. R.M.J. provided technical support for experiments and contributed to drafting the manuscript. J.L.R. conceived the project, designed the experiments, assisted in data analysis and contributed to drafting the manuscript.

**Additional Information**
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