Immunolocalization of deformed wing virus particles within the mite Varroa destructor

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Deformed wing virus (DWV) induces wing deformation when bees are infected during their pupal development. Field observations and laboratory experiments suggest that the mite Varroa destructor is a vector of the virus. Moreover, it has been stated that DWV replicates within this mite. In order to understand the role of V. destructor in the transmission of DWV, the objective of this work was to locate the sites of retention and/or replication of DWV within the mite by immunohistochemistry. There was no evidence that DWV was replicating in the mite as no tissues showed specific antibody binding to DWV. Also, there were no specific structures that could be suggested as retention sites. DWV was found only in the midgut lumen of V. destructor in structures resembling large, dense spheres, which were presumably faecal pellets.

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vacuum and the samples were left in fresh fixative overnight at 4 °C. The mites were then washed twice in 0.05 M sodium phosphate buffer for 10 min at room temperature. Dehydration of the mites was carried out using increasing grades of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 %) twice for 30 min at each concentration (the ethanol was renewed after the first 30 min period).

The mites were infiltrated with LR White acrylic resin (TAAB Laboratories Equipment) by immersion in increasing grades of resin, diluted with ethanol (v/v): 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % for two periods of 1 h in each concentration (the resin was renewed after the first period) at room temperature. Finally, they were transferred to fresh, pure LR White resin and the samples left overnight at 4 °C. The LR White resin was then replaced twice a day for 2 days. The mites were embedded in 100 % LR White resin in polypropylene capsules (TAAB Laboratories Equipment) and polymerized at 50 °C for 24 h.

Serial sagittal sections from one side of the mite to the other along the anterior/posterior plane across the body were cut with a glass knife using a Reichert-Jung ULTRACUT ultramicrotome. A series of microsections 2 μm thick was cut from all four mites and mounted. Each serial group was mounted on multitest eight-well Teflon slides (Erie Scientific Company) pre-coated with 1 % poly-d-lysine hydrobromide solution (Sigma-Aldrich) and heated overnight on a histological plate at 50 °C.

In order to prevent any non-specific binding by the primary DWV antibody (semi-purified IgG) to mite tissues, the sections were blocked with a solution of 0.01 M PBSTA [0.01 M PBS (Sigma-Aldrich), 1 % bovine serum albumen, 0.01 % Tween 20, 20 mM sodium azide; pH 7.2–7.4] and 10 % goat serum (Sigma-Aldrich) for 30 min at room temperature and then incubated overnight at 4 °C with the primary antibody diluted 1:300 in a mixture of 0.01 M PBSTA and 1 % goat serum. Mite sections were washed twice by immersing the slides in Copling jars filled with PBST (0.01 M PBS, 0.01 % Tween 20) for 10 min at room temperature. The sections were again blocked with PBSTA and 10 % goat serum for 30 min at room temperature and then incubated with the secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate; Sigma-Aldrich) diluted 1:50 in 0.01 M PBSTA and 1 % goat serum for 2 h at room temperature. Sections were washed twice in Copling jars filled with PBST for 10 min at room temperature. Finally, alkaline phosphatase substrate (SigmaFast; Sigma-Aldrich) was added to the sections for 25 min. To stop the reaction, the slides were immersed in Copling jars filled with distilled water.

Two sets of controls were used. The positive control was a section of the thorax of pupae injected with the virus to confirm the reliability and specificity of the method for detecting DWV. Sections from healthy, non-infested, non-infected pupae were used to confirm that there was no non-specific binding of the DWV antiserum to bee tissues. The negative control was used to demonstrate the specificity of the antiserum binding and to validate the results and consisted of sections of both mites and bee pupae. Both positive and negative controls were always included during the immunolabelling process of the sections.

In all sections (mites and honeybee pupae controls) where specific binding to DWV occurred, pictures were taken with a Leica digital camera adapted to a Zeiss Axioskop light microscope (×40 objective). To identify the tissues, the sections were stained with Harris’ haematoxylin (Sigma-Aldrich) for 1 h and washed with distilled water for 5 min. Sections were then counterstained with a 5 % solution of Eosin Y (Sigma-Aldrich) for 5 min and rinsed with distilled water for 5 min.

The use of the goat anti-rabbit enzyme conjugate to identify areas where the specific DWV antibody had bound to the virus antigens proved to be both sensitive and specific. The reaction between alkaline phosphatase and the substrate produced a strong red-coloured signal at the site where specific DWV antibody binding had occurred. Even after counterstaining with Harris’ haematoxylin and Eosin Y, the signal could be differentiated from the colour produced by counterstaining.

In all sections probed with the antiserum and examined, specific binding to DWV was only visualized and located in the lumen of the gut, primarily in the anterior, middle and posterior caeca, and in the central ventriculus of the midgut (Fig. 1a–c). Large aggregates of virus gave very strong signals, but small clumps of particles could also be clearly differentiated.

The post-colon, the distal part of the midgut, also showed specific antibody binding to DWV in the lumen, but in this case the signal was detected in structures that looked like large, dense spheres, which were presumably faecal pellets (Fig. 1a, c and Fig. 2c, d). The epithelial cells of the midgut wall were clearly distinguishable and no specific antibody binding to DWV particles was detected within or on the surface of these cells (Fig. 2b, c) in any of the sections.

Reproductive structures, such as the ovary with developing eggs and the lyrate organ, were clearly differentiated by counterstaining, but specific antibody binding to DWV particles in these tissues was not observed (Fig. 2e, f). Specific binding to DWV was observed in one mite in the lumen of the oesophagus (Fig. 2a). No signal was detected in other structures such as muscles, salivary glands and Malpighian tubules or in any other tissue.

The colour reaction produced by the alkaline phosphatase substrate was very specific; it was only observed when DWV was present in the thorax sections of infected pupae. No virus was detected in thorax sections of uninfected bees, even when using the specific primary antibody against DWV.

No signal was detected in the negative controls of the bee thorax and mite sections tested (Fig. 1b, d and f).

Using immunohistochemistry, the lumen of the midgut was identified as the only site of DWV accumulation in V.
No mite organs, tissues or cells showed specific binding to the DWV antibody. These results agree with the results obtained by Zhang et al. (2007) who found virus particles, probably DWV, only in the gastric caecal lobes of the midgut using a similar technique.

The fact that DWV was localized in the midgut is an indication that the virus is ingested with the haemolymph of an infected bee or pupa. It is likely that DWV accumulates and may be retained in the gut together with the ingested food, as this is the main site of digestion and absorption in mites (Evans, 1992). Thus, the question still remains of how these virus particles in the midgut are transferred to another bee host in sufficient quantity to cause infection. If virus particles are only found in the lumen of the midgut, it seems unlikely that efficient DWV transmission occurs during mite feeding activities, as regurgitation is said not to occur in Gamasida, the order to which *V. destructor* belongs (Evans, 1992).

An alternative means of virus transmission could simply be by contamination of the mouthparts, as mites perforate thin areas of the integument of adult bees or pupae with toothed chelicerae to feed on the haemolymph. There is a probability that virus particles from systemically infected hosts would be retained externally on the mouth parts or in

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**Fig. 1.** Sagittal sections of *V. destructor* showing detection of DWV antigen. The reaction between alkaline phosphatase and substrate produced a strong red colour where the specific DWV antibody bound to the antigen (a, c, e). Controls are shown in (b), (d) and (f) where specific antibody against DWV was omitted. CA, Caecum; DO, developing oocytes; LG, lateral gland; LO, lyrate organ; PC, post-colon. Bars, 10 μm.
the ducts that lead into the pharynx. When mites feed on the next bee or pupa, the virus could be inoculated into the new host by penetration of the mouth parts. However, this mode of transmission seems less likely, as such vectors lose the ability to infect new hosts soon after the virus acquisition period (Nault, 1997). Moreover, evidence from transmission experiments with different honeybee viruses has shown that *V. destructor* is able to transmit to several successive pupae without virus reacquisition (Wiegers, 1986; Ball, 1989; Santillán-Galicia, 2006). This suggests a means of transmission other than surface contamination of the mite mouthparts.

The close taxonomic relationship between *V. destructor* and ticks (vectors of vertebrate viruses) (Evans, 1992), and the fact that both feed on the lymph of their host, may suggest that *V. destructor* transmits DWV and possibly other viruses by similar mechanisms in which the virus replicates in midgut cells and then passes to the salivary glands where it is transmitted by feeding (Nuttall *et al.*, 1994). If this mechanism is used by *V. destructor* to transmit DWV, it would explain the replication of DWV within the mite, as suggested by Ongus *et al.* (2004) and Yue & Genersch (2005) who reported the replication of DWV in *V. destructor* using PCR by detecting the negative strand of the genome of DWV.

**Fig. 2.** Mite sections showing specific DWV antibody binding in the midgut: (a) lumen of the oesophagus; (b) midgut’s caecum; (c, d) post-colon; (e, f) ovary. CA, Caecum; DO, developing oocytes; EC, epithelial cells of the wall of the midgut; FP, faecal pellets; LO, lyrate organ; O, ovary; OE, oesophagus; PC, post-colon. Bars, 2 μm.
in the mites. However, in the present histological study, there was no evidence of virus replication in the mites because no DWV was detected within any of the cells, organs or tissues. There are two reasons that could explain these results. The first may be that the mites sectioned did not acquire sufficient DWV particles to initiate replication and infect tissues or organs, as suggested by Rehacek (1965), who stated that a minimum concentration of the agent is required for its further development in the vector.

The second possible reason is the sensitivity of the technique for detection of virus particles. PCR techniques are more sensitive than serological techniques for the detection of a few virus particles. Moreover, light microscopy did not allow us to determine whether there were any virus particles within the cells, so this evidence may have been missed. For example, Zhang et al. (2007) did not find any virus particles in any organ or tissue within the mite other than the midgut using light microscopy, but with electron microscopy they found numerous virus particles in the cytoplasm of gastric caecal cells, suggesting virus replication in V. destructor.

Although molecular techniques can be more sensitive and may suggest replication of the virus (Yue & Genersch, 2005), the immunohistological technique used here can still be useful to detect the virus in specific tissues or organs within the mite. Further insight at a ultrastructural level still be useful to detect the virus in specific tissues or organs. There are two reasons that could explain these results. The first is that the amount of virus acquired by the mite may have been missed. For example, Zhang et al. (2007) did not find any virus particles in any organ or tissue within the mite other than the midgut using light microscopy, but with electron microscopy they found numerous virus particles in the cytoplasm of gastric caecal cells, suggesting virus replication in V. destructor, which is directly related to DWV prevalence in the bee population.

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