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ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologic

RNA viruses in the house dust mite *Dermatophagoides pteronyssinus*, detection in environmental samples and in commercial allergen extracts used for *in vivo* diagnosis

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Abstract

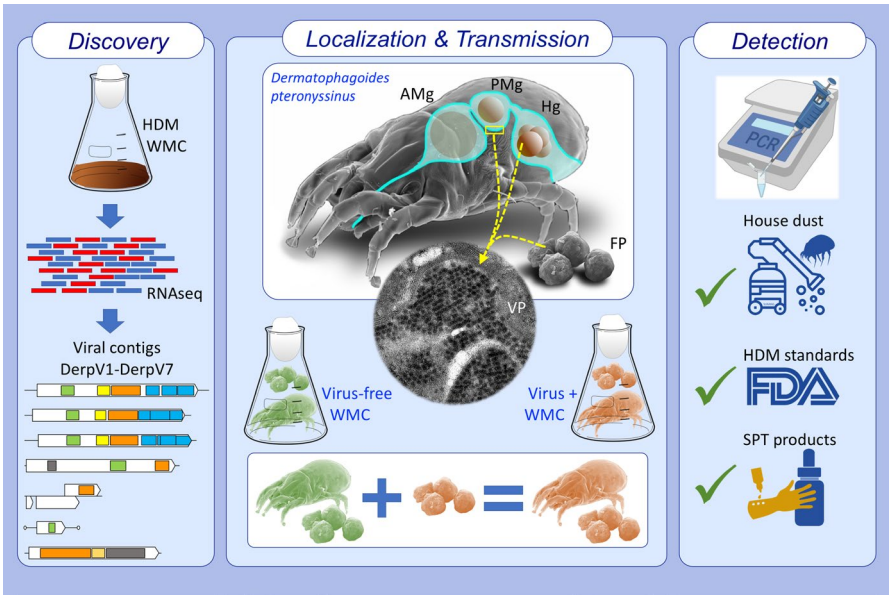
Background: Allergy to house dust mites (HDM), the most important source of indoor allergens worldwide, is diagnosed and treated using natural extracts from cultures that can contain immunoactive components from the HDM microbiome, including mite-infecting viruses. This study aimed to contribute to the discovery and characterization of RNA viruses from *Dermatophagoides pteronyssinus*, followed by their detection in different mite-derived sources.

Methods: Viruses were assembled after *in silico* metatranscriptomic analysis of *D. pteronyssinus* RNA samples, visualized by electron microscopy, and RNA detected by direct RT-PCR or data mining. Mite culture performance was evaluated *in vivo*.

Results: Seven RNA viruses were identified in our laboratory stock colony. Picornavirus-like viral particles were detected in epithelial cells of the digestive system and in fecal pellets. Most of these viruses could be persistently transmitted to an inbred virus-free colony by inoculating fecal material from the stock colony. Upon viral infection, no significant effect could be seen on mite population growth. Transcriptomic screening confirmed the presence of homolog sequences to these viruses in independent laboratory stocks of *D. pteronyssinus* and in other Astigmata mites. Noteworthy, RNA from most of the viruses could be detected by RT-PCR on house dust samples, reference standards, and/or commercial diagnostic *D. pteronyssinus* extracts.

Conclusions: Our results show that viral infections are common and widespread in *D. pteronyssinus*, both in natural and culture-based growth conditions. Potential effects on the mites themselves and consequences toward allergenicity in humans whether exposed naturally or after immunotherapy are discussed.

KEYWORDS
Entomopathogens, immunotherapy, skin-prick test, transcriptome, viral infection



GRAPHICAL ABSTRACT

Seven RNA viruses were identified in the transcriptome of *D. pteronyssinus*, being picorna-like viruses the most abundant. Viral particles are found in epithelial cells of the digestive system, and in fecal pellets from which they can be horizontally transmitted to other virus-free mites. Viral RNA was detected by RT-PCR on house dust samples, reference standards, and allergy-related pharmaceutical products.
Abbreviations: AMg, anterior midgut; FDA, food and drug administration; FP, fecal pellet; HDM, house dust mite; Hg, hindgut; PMg, posterior midgut; RNAseq, RNA sequencing; SPT, skin-prick test; VP, viral particles; WMC, whole mite culture.

1 | INTRODUCTION

House dust mites (HDM) are considered the most important source of indoor allergens worldwide, leading to perennial rhinitis and asthma, and worsening atopic dermatitis.[1] One of the major producers of house dust allergens and the most widespread species is *Dermatophagoides pteronyssinus*. The main route of exposure to HDM allergens is via their fecal particles, which become easily airborne.[2] Allergy to HDM is currently treated by allergen-specific immunotherapy (AIT) using natural allergen extracts produced from mite-derived fractions obtained in large-scale commercial cultures. These extracts are very complex since they include biological components from both the mites and their associated microbiomes.[3,4] Noteworthy, mite cultures commonly contain immunoactive endotoxins of bacterial origin such as liposaccharide (LPS) that are systematically controlled as per pharmacological certifications.[5,6]

During the last decade, viral metagenomics hand in hand with breakthrough mining techniques on high throughput sequencing data are revealing the extraordinary diversity and ubiquity of viruses in the biosphere.[7] A paradigmatic example is the unprecedented number of novel virus species being discovered in arthropods, showing genomic structures and phylogenetic relationships exceeding by far our previous knowledge on invertebrate RNA viruses.[8] In the case of mites, arachnids of the *Acari* sub-class also including ticks, viral metagenomic research is still in an incipient stage. Some notable exceptions are studies carried out on species of economically important impact, such as the honeybee ectoparasite *Varroa destructor*,[9,10] the phytophagous pest *Tetranychus urticae*,[11] and, very recently, the medically important mites *D. pteronyssinus*, *Dermatophagoides farinae*, and *Tyrophagus putrescentiae*.[12]

Expanding our understanding on the viruses associated to HDM is of medical interest. Commercial HDM cultures may be infected with non-human infecting viruses that make their way to the finished pharmaceutical products used to diagnose and treat allergy. This could have unknown consequences for both the mite culture and the exposed human subject, which should be considered in terms of safety and/or efficacy. In the case of mites, viruses could affect the culture performance or the production of allergens; in turn, in humans, they might trigger immunological or inflammatory responses. Although not expected to show pathogenic effects on humans, some components of the mite-derived virions could show antigenic properties or elicit innate immune responses that further interact with the allergic response, as has been demonstrated for pathogenic viruses.[13,14] Interestingly, the inclusion of inactive viral-like particles as an adjuvant in AIT products has been suggested due to their immunomodulatory effects.[15,16] Additionally, the study of mite-pathogenic viruses could eventually be promising to develop new biocontrol methods for HDM.

Herein, we report the discovery and characterization of seven RNA viruses in *D. pteronyssinus*. We detect the presence of viral RNA in different in-house laboratory colonies by direct molecular methods. We localize picornavirus-like particles in the digestive system and fecal pellets of the mite by electron microscopy. Additionally, we demonstrate that fecal-borne viruses can be a way of horizontal transmission between mites, and we gain insight on the effects of viral infection over mite population growth. Finally, by *in silico* screening and direct molecular methods, we

confirm the presence of homolog sequences to these viruses in other sources beyond our laboratory colonies, including different laboratory stocks of *D. pteronyssinus* and other Astigmata mites, samples from house dust containing wild populations of *D. pteronyssinus*, and in pharmaceutical products used to diagnose HDM allergy.

2 | MATERIALS AND METHODS

2.1 | Mite cultures

Our *D. pteronyssinus* stock colony derives from a commercial culture[17,18] and was established in our laboratory in 2012. Inbred *D. pteronyssinus* colonies were obtained in a former inbreeding project by initial mating of adult virgin couples from the stock colony followed by consecutive brother-sister sib-mating.[19] Standard culture conditions and sampling of body and fecal fractions are described as supporting information (Text S1, Figure S1).

2.2 | Environmental samples, reference standards, and commercial products

House dust environmental samples were vacuum-collected in mattresses of HDM-sensitized subjects in Northwestern Spain, a region where the prevalence of *D. pteronyssinus* is well documented.[20] Samples from Galicia were collected at the Santiago de Compostela area, and samples from Asturias were collected at Infiesto and Villamayor municipalities. The occurrence of *D. pteronyssinus* in house dust samples was determined by specific molecular methods based on the amplification of ribosomal DNA (rDNA) (Text S1). United States Food and Drug Administration (FDA) reference *D. pteronyssinus* extracts E10-Dp, E11-Dp, and E12-Dp were provided by the Center for Biologics Evaluation and Research (CBER, Silver Spring, USA). The World Health Organization and International Union of Immunological Societies (WHO/IUIS) *D. pteronyssinus* international standard 82/518 was provided by the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). The following commercial *D. pteronyssinus* skin-prick testing products from four international suppliers available in the Spanish market were analyzed (in alphabetic order): ALK-Abelló (Madrid, Spain; batch A7438_CT01), LETIPharma (Tres Cantos, Spain; batch 20985617), Roxall Medizin (Zamudio, Spain; batch TCDPT 191115), and Stallergens-Greer (Barcelona, Spain; Alyostal batch 9717109). Note that the results have been shown in the manuscript by using a code in a random order instead of the name of the company (ie, company 1–4).

2.3 | *In silico* analysis of viruses

Viral RNA sequences were identified in unmapped reads resulting from the alignment of in-house *D. pteronyssinus* WMC RNAseq libraries (four replicates) against the *D. pteronyssinus* genome assembly ASM190122v2 (RefSeq accession GCF_001901225.1).[21] Specific methods to assemble and characterize viral sequences, together with the screening of public Astigmata transcriptomes and phylogenetic analyses, are detailed as supporting info (Text S1).

2.4 | Detection of viral RNA by reverse transcription and PCR

The presence of viral RNA in WMC, growth medium, mite body fraction, fecal fraction, reference standards, commercial products, and environmental samples was assessed by RT-PCR. Detailed methods for RNA extraction, reverse transcription and PCR, including to the amplification of a picornavirus (DerpV3) negative (antigenomic) strand, are described as supporting information (Text S1, Table S1).

2.5 | Virus visualization by electron microscopy

The localization of virus particles in *D. pteronyssinus* tissues was studied by Transmission Electron Microscopy (TEM). Virion morphology was inspected by negative staining, and fecal pellets were visualized by Scanning Electron Microscopy (SEM). See Text S1 for detailed methods.

2.6 | Transmission of viruses to *D. pteronyssinus*

An inbred virus-free *D. pteronyssinus* colony (free of the seven identified viral genotypes as determined by RT-PCR) was inoculated with virus particles derived from the fecal material of the stock colony. The inoculated colony was established and characterized by RT-PCR to detect viral acquisition. Additional culture bioassays were conducted to determine the effects of viruses over mite growth. See Text S1 for methodological details.

3 | RESULTS

3.1 | Virus discovery

Our analysis identified seven RNA viral contigs within a transcriptome built from unmapped reads obtained from the alignment of *D. pteronyssinus* WMC RNAseq libraries against the NCBI´s RefSeq genomic assembly of the species.[21] BLASTx against the NCBI GenBank´s "RNA viruses" database showed protein identities ranging from 22.8 to 99.1% (BLASTx best hits; Table S2). The identified viral contigs were named *Dermatophagoidespretonyssinus* virus 1–7, DerpV1 to DerpV7 (Genbank accessions MW355885 to MW355891). Six out of the seven contigs showed similarity to (+)ssRNA viruses: DerpV1/2/3, that were related to the order *Picornavirales*; DerpV4, to the family *Togaviridae*; DerpV5, to the families *Tombusviridae*

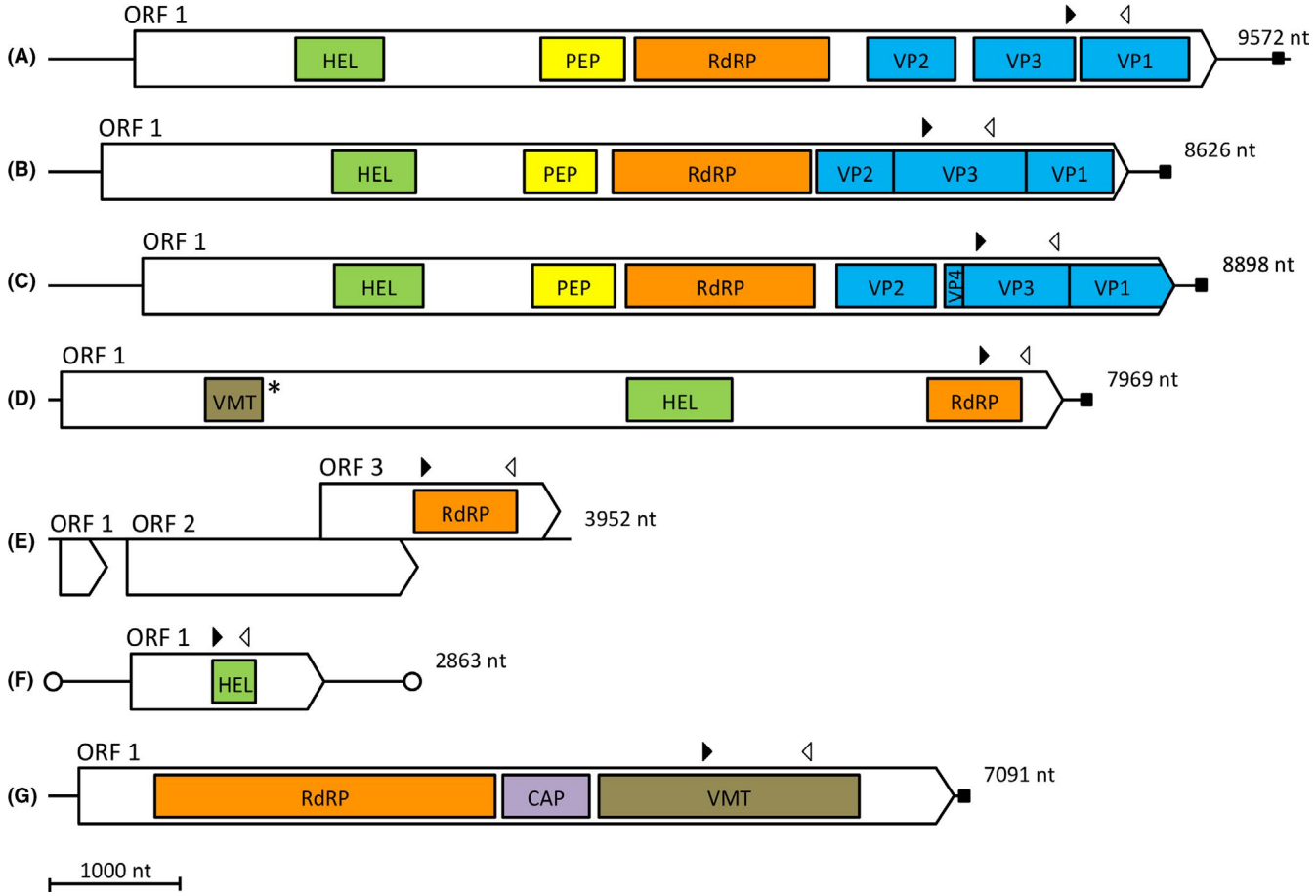


FIGURE 1 Genomic schematic representation of the RNA viruses identified in this study. (A) DerpV1 (GenBank accession MW355891); (B) DerpV2 (MW355890); (C) DerpV3 (MW355889); (D) DerpV4 (MW355888); (E) DerpV5 (MW355887); (F) DerpV6 (MW355886); (G) DerpV7 (MW355885). Positive strands are represented (5' to 3'). Arrows indicate open-reading frames (ORF); directions correspond to the orientation of the respective gene. Putative conserved domains are shown as rectangles, an asterisk denotes protein prediction could not established based on structure (Phyre2), but only on InterPro database search. Black squares show poly(A) tails. Empty circles at terminus indicate linearization of a circular molecule. Black and empty triangles show positions of forward and reverse PCR primers, respectively. Acronyms: CAP (mRNA-capping region of the RdRP); HEL (viral RNA helicase); PEP (peptidase C3); RdRP (RNA-dependent RNA polymerase); VMT (viral methyltransferase); VP# (capsid viral proteins)

TABLE 1 Presence of viral-like RNAseq reads in *D. pteronyssinus* in-house transcriptomes

RNAseq libraries[a]	Average total reads/library	Alignment rates (%)[b]			
		To Dp RefSeq assembly	Unmapped reads to viruses	Reads unmapped to Dp or viruses (%)[c]	Reads corresponding to viruses (%)[c]
WMC (n = 4)	30,929,331	47.5 ± 1.3	75.1 ± 0.8	12.2 ± 0.1	40.4 ± 1.3
Females (n = 3)	24,313,936	72.6 ± 0.6	48.6 ± 0.9	14.4 ± 0.2	13.1 ± 0.5
Males (n = 3)	25,108,996	51.1 ± 4.6	61.4 ± 3.6	18.7 ± 0.3	30.2 ± 4.3
Nymphs (n = 3)	24,677,562	70.6 ± 3.1	54.7 ± 4.8	13.1 ± 0.1	16.3 ± 3.1
Larvae (n = 3)	22,281,857	64.6 ± 2.7	62.1 ± 3.3	13.4 ± 0.3	22.0 ± 3.0

Note: Alignment rates were determined, first, by aligning raw RNAseq reads to the RefSeq genomic assembly ASM190122v2 (accessionGCF_001901225.1), and, second, after aligning the resulting unmapped reads to the viral RNA sequences identified in this study (DerpV1-7). Figures are averages ±SEM. "Dp" denotes *D. pteronyssinus*; "viruses" refers to the fasta file containing all seven viral contigs from this study.

[a]RNAseq libraries were obtained using total RNA extracted from stock whole mite cultures (WMC; n = 4), and from pooled samples of purified bodies at different developmental stages: females (n = 3); males (n = 3); nymphs (n = 3), larvae (n = 3).

[b]"Alignment rate" denotes the percentage of RNAseq reads being mapped to a reference after HISAT2 alignment.

[c]Calculated percentages of RNAseq reads with respect to the total reads.

and *Nodaviridae*; and, DerpV6, that could not be related to any viral family. In addition, one contig, DerpV7, could be associated to (–) ssRNA viruses of the family *Chuviridae*. See Figure 1 for a representation of their genomic structure and conserved domains. Further analyses for each viral contig regarding sequence similarity and genomic features are detailed in Text S2, Figure S2, Table S2 and S3. Interestingly, the alignment of our *D. pteronyssinus* RNAseq libraries from total RNA of WMC against the *D. pteronyssinus* RefSeq genome assembly resulted in low average alignment rate of 48% (Table 1). The alignment of the resulting unmapped reads to a fasta file containing our seven viral sequences revealed that approximately 75% of these corresponded to viruses. Overall, only 12% of the reads did not align either to the *D. pteronyssinus* genome or to viruses, hence at least 40% of the total reads corresponded to viruses. As the RNAseq libraries were obtained from WMC, including diet and fecal pellets, we additionally analyzed the presence of viruses on RNAseq libraries obtained from purified bodies of females, males, nymphs, and larvae. Virus-matching reads were found in all developmental instars and adults, but the percentage of reads corresponding to viruses on those libraries was lower (13–30%) than on WMC (Table 1). Finally, the coverage (ie, abundance) of each virus was estimated by total-read-base-counts (ie, the sum of per base read depths) using BedCov, being DerpV3 the most abundant RNA virus in all samples followed by DerpV2, DerpV1, and DerpV4; the less abundant were DerpV5, DerpV6, and DerpV7 (Table S4).

3.2 | Molecular detection of viral RNA in *D. pteronyssinus* laboratory colonies

A multiplex RT-PCR method was designed for the simultaneous detection of six of the viruses, DerpV1 to 6. For virus DerpV7, a singleplex PCR strategy was adopted due to its undetectable amplification by multiplex PCR. As expected, all seven viruses were detected using these methods on RNA from WMC, containing bodies and fecal pellets (Figure S3). In a next step, the method was applied to detect viruses in RNA samples from purified bodies from our stock colony, and from twelve other *D. pteronyssinus* colonies established previously after a sib-mating inbreeding program started out from different mite couples of the stock.[19] Interestingly, none of the inbred colonies contained all seven viruses identified on the stock colony, indicating that the isolation of mite couples during the inbreeding program had influenced viral transmission. DerpV1 was detected in colonies deriving from the original couple "I"; DerpV1/4 in colonies from "O"; DerpV2/3/4 in colonies from "C"; and no viral RNA was detected in colonies from couples "J" and "M" (Table S5). DerpV7 could not be detected in samples of purified bodies from any of the colonies, including the stock colony. The detection of viral RNA in fecal samples by RT-PCR was only achieved for DerpV3 after applying a modified singleplex protocol (10-fold less RT template) potentially due to inhibition from the fecal RNA extract (data not shown). In addition, for DerpV3, the most abundant virus, the intermediate replicative form (antigenomic RNA, negative strand) could also be detected in samples from WMC and purified bodies from the stock colony using a RT-PCR strategy of high specificity (Figure S4). Finally, attempts failed to detect any of the viruses in RNA extracts from the culture growth medium.

3.3 | Detection of virus particles by electron microscopy

Virus particles of about 30 nm in diameter and near-hexagonal/octagonal shape were detected in the digestive tract of *D. pteronyssinus*, while they could not be found in other tissues, such as the nervous system, muscle, fat body, or gonads. They were specifically located in the cytoplasm of epithelial cells of the midgut. The distribution of viral particles could vary from scattered in the cytosol, including microvilli, enclosed in vesicle-like structures, or forming dense paracrystalline arrays often associated to host cellular membranes (Figure 2A,B,C). Virus particles could also be found at high density in the gut lumen, within non-excreted fecal pellets, specifically into what appeared to be cell remnants shed from the gut epithelium (Figure 2D,E,F). In addition, viral particles were detected by negative staining electron microscopy in fecal pellets directly purified from WMC (Figure 2G,H). No other morphologically distinct viral particles, potentially corresponding to different virus species, were identified.

3.4 | Transmission of RNA viruses and effects on mite population growth

In order to assess the potential of feces as a route of transmission of viruses to other mites, fecal pellets purified from the virus-containing stock colony were used to inoculate mites separated from the inbred colony J-1–1–1–1, which was free of the seven viruses identified in this study (ie, virus-free; Table S5). After two and six culture cycles since the initial fecal spike (81 and 268 days, respectively), the presence of viral RNA in the inoculated and the original inbred colonies was assessed by multiplex RT-PCR, results are shown in Figure 3. As expected, none of the studied viruses was detected in the original non-inoculated J-1–1–1–1 colony. However, positive detection of viral RNA was observed for DerpV1/3/5/6 by multiplex PCR on inoculated J-1–1–1–1 WMC, as well as on separated bodies from this colony, showing the same results after two and six culture cycles. The presence of DerpV2, but not DerpV4 or DerpV7, was further confirmed by singleplex RT-PCR in all samples from the inoculated colony. Additionally, the overall effect of the identified viruses on the fitness of *D. pteronyssinus* was evaluated by comparing the growth of small-scale populations from adults of the original virus-free inbred colony and from the inoculated colony. Mite population numbers after 3 weeks increased over 10-fold for both colonies, but no significant difference was recorded between cultures on neither total mites ($p = .169$; Figure 4A), nor the percentage of laid eggs ($p = .066$; Figure 4B). Likewise, no significant difference was found on the duration of the standard culture cycle in flasks ($p = .452$; Figure 4C).

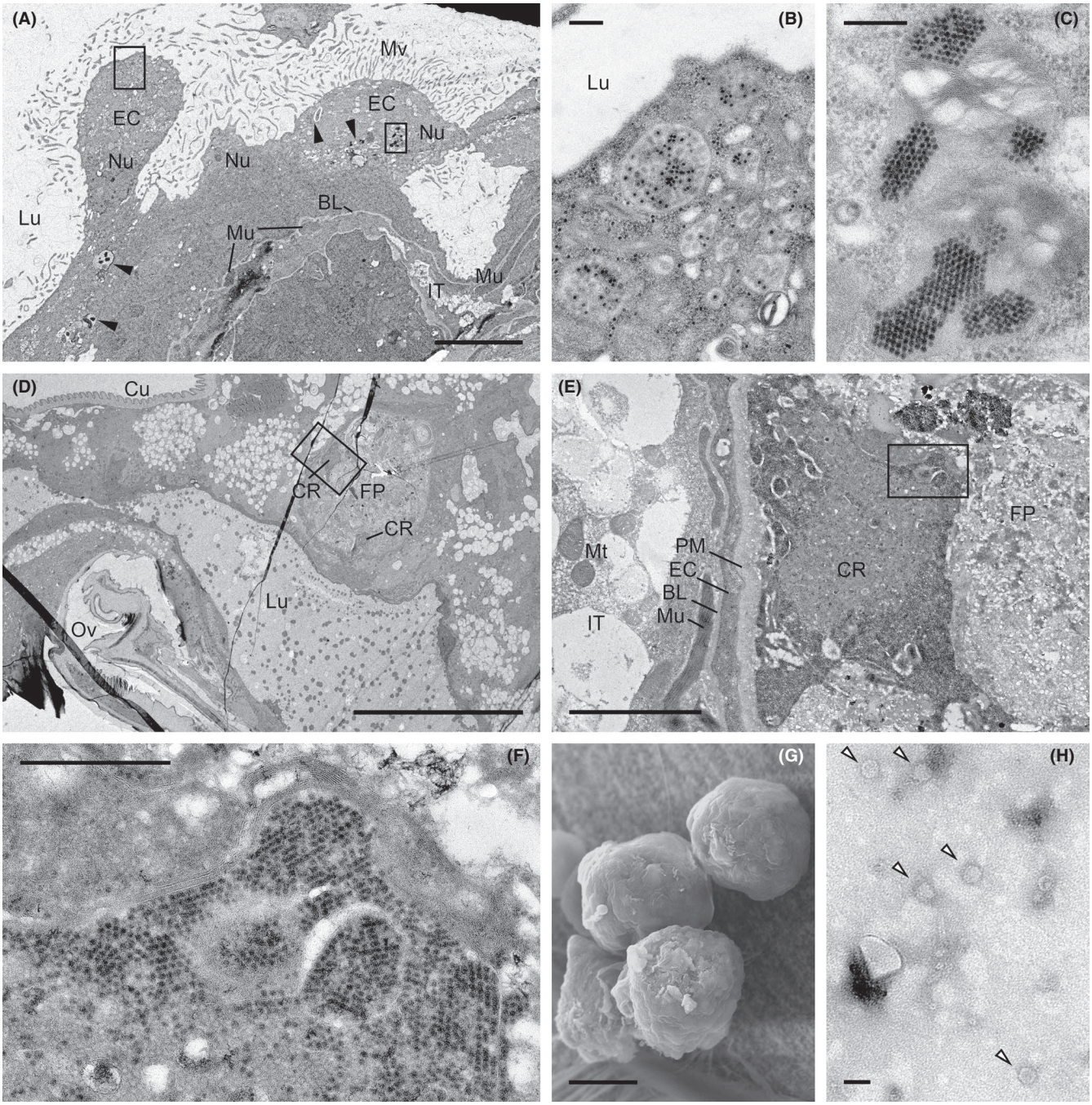


FIGURE 2 Detection of virus particles in the digestive system of *D. pteronyssinus* by electron microscopy. (A) Histological section showing digestive epithelial tissue with the occurrence of virus particles; high dense pockets of particles are indicated with arrowheads and rectangles (close-ups available in panels B, C). (B) Virus particles scattered in the cytoplasm or in association to vesicle-like enclosures. (C) Paracrystalline arrays of virus particles. (D) Histological section of an adult female showing the posterior midgut bearing a fecal pellet (top-right quadrant). The fecal pellet encloses cell remnants containing virus particles. The area corresponding to the rectangle is magnified in panel E; note it was obtained from an adjacent histological cut. (E) Digestive cell remnant heavily infected with virus particles, rectangle close-up in panel F. (F) Virus particles arranged in paracrystalline arrays, note membrane-like structures delimiting the area of infection. (G) Scanning electron micrograph of purified fecal pellets. (H) Negative staining electron micrograph of virus particles released from fecal pellets; white arrowheads indicate individual virions, note their polygonal contour. BL, basal lamina; CR, digestive cell remnant carrying virus; Cu, exocuticle; EC, gut epithelial cell; FP, fecal pellet; IT, intermediate tissue; Lu, gut lumen; Ov, ovipositor; PM, peritrophic matrix; Nu, nucleus; Mt, mitochondrion; Mu, visceral muscle; Mv, microvilli. Scales: A = 5 µm; B = 200 nm; C = 200 nm; D = 40 µm; E = 3 µm; F = 500 nm; G = 20 µm; H = 50 nm

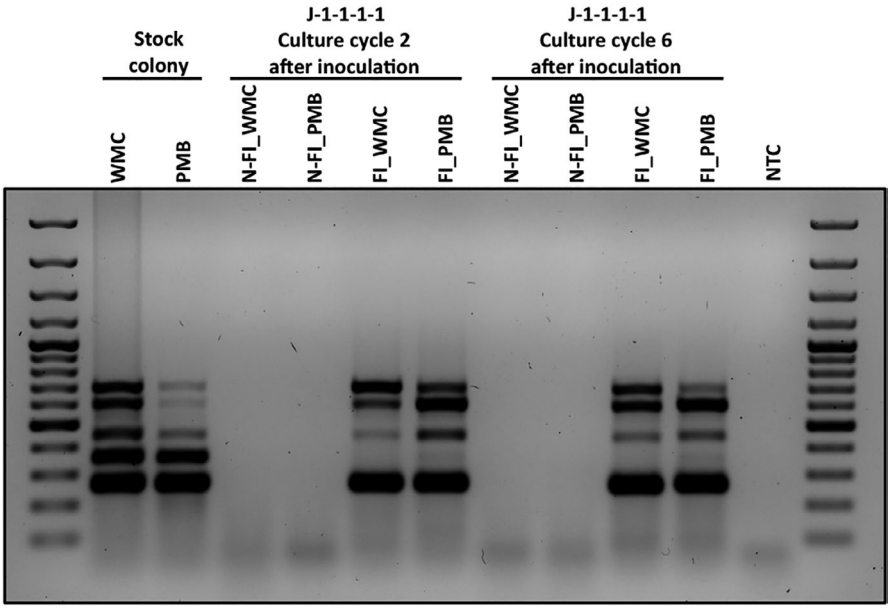


FIGURE 3 Fecal-mediated transmission of RNA viruses from the stock *D. pteronyssinus* colony into the inbred colony J-1–1–1–1. Six virus species (DerpV1-6) were screened by multiplex RT-PCR and agarose gel electrophoresis. RNA samples were extracted from both whole mite cultures (WMC) or purified mite bodies (PMB). J-1–1–1–1 colonies were sampled at the 2nd and 6th culture cycle after the initial inoculation with fecal pellets from the stock colony; both non-inoculated (N-FI) and inoculated (FI) cultures were analyzed. A no template control was included (NTC). The molecular-size marker was GeneRuler™ 100 bp Plus. Amplicon sizes are (in decreasing order; use lane "WMC" as reference): 716 bp (DerpV5), 622 bp (DerpV3), 549 bp (DerpV2), 468 bp (DerpV1), 372 bp (DerpV4), 271 bp (DerpV6)

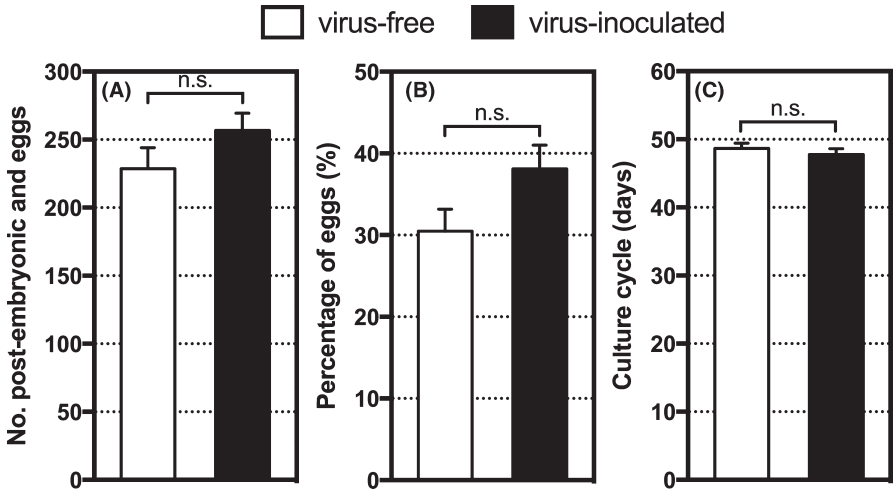


FIGURE 4 Impact of the inoculation of viruses in the population growth of a *D. pteronyssinus* colony. The inbred colony J-1–1–1–1, reported as virus-free by molecular methods, was inoculated with fecal-derived viruses from the stock colony, *virus-inoculated*. The growth parameters of both colonies were compared. (A) Total mite population after 3 weeks of small-scale culture (n = 14 microtubes). (B) Percentage of non-hatched eggs in small-scale populations after 3 weeks (n = 14). (C) Duration of the standard-scale culture cycle (in 2 g flasks; n = 9 cycles; first cycle not included). Columns indicate mean values; errors bars indicate standard errors of the mean; means were statistically compared by unpaired t test (n.s. denote "not significant," $p > .5$)

3.5 | Detection of viral RNA in other sources

Nine environmental samples obtained from house dust were screened. First, the occurrence of *D. pteronyssinus* was detected by rDNA amplification, showing detectable levels in seven out of the nine samples. Then, viral presence was assessed by RT-PCR, showing two positive samples for at least one virus, including DerpV1 and DerpV2 (Table 2; Figure S5). Additionally, four commercial allergy diagnostic products and four international reference standard extracts from *D. pteronyssinus* were analyzed by RT-PCR. Remarkably, all the pharmaceutical products and the three CBER/FDA standards were positive for at least one virus (Table 2; Figure S5). Likewise, RNA from all the virus species identified in this study, except for DerpV7, could be detected in at least one of the commercial extracts

TABLE 2 Detection of *D. pteronyssinus*-derived viral RNA in allergy international standards, commercial pharmaceutical products, and house dust samples by RT-PCR

	International reference standards				Commercial pharmaceutical products				House dust samples							
	CBER/FDA E10-Dp	CBER/FDA E11-Dp	CBER/FDA E12-Dp	WHO/IUIS 82/518	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Asturias 1	Asturias 2	Asturias 3	Galicia 1	Galicia 2	Galicia 3	Galicia 4	Galicia 5	Galicia 6
DerpV1	+[b]	+[b]	+[b]		+[b]	+[b]	+[b]						+[b]		+[b]		
DerpV2		+[b]											+[b]				
DerpV3	+[b]	+[b]	+[b]		+[b]	+[b]											
DerpV4		+															
DerpV5		+						+									
DerpV6					+	+		+									
DerpV7																	
rDNA Dp[a]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+			+	+	+	+	+	+

[a]The presence of *D. pteronyssinus* (Dp) in house dust samples was assessed by the specific amplification of ribosomal DNA (rDNA). N.a. denotes "not analyzed".

[b]RT-PCR products were analyzed by Sanger sequencing with forward and reverse specific primers.

or international standards. The two more prevalent viruses were DerpV1 and DerpV3, with six and five positives out of the eight analyzed samples, respectively. Direct sequencing of amplification products for DerpV1/2/3 from all different origins revealed that each individual sample exhibited a distinct variant on the amplified region (468, 549, and 622 bp, respectively; Table S1, Figure 1), with sequence identities to our assembled viral sequence ranging from 95.3–97.0%, 95.1–95.3%, and 92.1–94.2%, respectively (Figure S6,S7,S8). In order to further explore the presence of viruses similar to those in this study, public transcriptome databases were additionally screened revealing the occurrence of homologous sequences to DerpV1/3/4/6/7 in two other *D. pteronyssinus* laboratory stock cultures (accessions SRX2026624, SRX4482484)[22,23] (Table S6). Besides, similar sequences to DerpV1/3/6 were also found in RNAseq data from the HDM *D. farinae* and the storage mite *T. putrescentiae*, albeit their coverage was insufficient to assess viral similarity between host species, except for one case. Alignment of RNAseq reads suggested the occurrence of a DerpV1-like virus in *T. putrescentiae* (approximately 88% of DerpV1´s sequence with 91% identity). Finally, no sequence similarity was found in other mite species of the order Astigmata.

4 | DISCUSSION

The virome of *D. pteronyssinus* resolved in this study comprised seven different viral contigs, all of them corresponding to RNA viruses. RNA sequences associated to the expression of putative DNA viruses have not been identified in our analysis. Most of the identified viruses show relationship with arthropod-infecting taxa and/or their closest virus genotypes are associated to arthropods or invertebrates, but not to mammals. Five out of the seven viral contigs showed >95% protein sequence identity to viral accessions from a similar *D. pteronyssinus* meta-transcriptomic survey published during the preparation of this manuscript.[12] The similarity of DerpV6 and DerpV7 to known viruses in databases was very low, suggesting they are putatively novel (extended details in Text S2). The three most abundant viruses, DerpV1/2/3, were related to the order *Picornavirales*. Among them, DerpV1 and DerpV3 showed capsid domains with structural homology to those from formal members of the picornavirus family *Dicistroviridae*, as well as phylogenetic relationship to informally classified dicistro-like viruses. However, their monocistronic genomic organization differs from all currently assigned dicistroviruses, which are bicistronic with separate nonstructural and structural ORFs.[24] Our results highlight the still limited knowledge that we have on arthropod ssRNA viruses, which, as we are starting to discover, are remarkably more diverse than what depicted in current taxonomic classifications.[8,25,26] Multi-viral infections, as our results for *D. pteronyssinus* suggest, are likely to be the norm rather than the exception based on recent studies on other mites and arthropods.[8–12] Nonetheless, since in our study mite subpopulations were sampled instead of individual mites; at this stage, it is impossible to ascertain whether the identified viruses coexist

in simultaneous infections within single individuals, or if they occur separately in different mite hosts of the population. Our finding that distinct mite inbred lines (obtained by recurrent crosses of single isolated couples) exhibited different, or even absent, viral content suggests that single individuals from the stock population might show heterogeneous viral profiles, although the underlying reason remains unknown.

The viral incidence reported in our meta-transcriptomic study (up to 40% viral sequences in WMC transcriptomes), yet being high, is not uncommon when compared to other transcriptomic surveys conducted on HDM and other invertebrates.[8,12] This result, together with the fact they were detected in all the studied developmental stages, primarily suggested that the occurrence of some of these viruses was a result of a persistent infection of the host. Further experimental data, including the visualization of infected cells by TEM, the controlled infection of a virus-free colony with viruses from the stock colony, as well as the molecular detection of DerpV3´s antigenomic negative strand do unequivocally demonstrate the active viral replication in *D. pteronyssinus*. Electron microscopy observations revealed the presence of viral particles in epithelial cells of the *D. pteronyssinus* midgut and in fecal pellets. The accumulation of viruses in fecal pellets is consistent with our RNAseq data, with WMC samples (high fecal content) exhibiting higher proportion of RNA viral sequences than purified body samples (13–30%). A single viral morphological type was observed by TEM, icosahedral-like particles of approximately 30 nm, sometimes showing paracrystalline arrangement associated to host cellular membranes. Altogether, these features were highly consistent with that described previously in picorna-like viruses of families *Dicistroviridae* and *Iflaviridae*, which are known to infect arthropod hosts[24,27,28] and would be in accordance with the visualization of DerpV3, a picorna-like virus possibly related to the family *Dicistroviridae* and by far the most abundant virus reported in our study.

RNA from the studied viruses could not be detected by RT-PCR in the growth medium used to rear our colonies, suggesting that the mite´s food was not the origin of the viruses, but probably the wild mites originally collected to start the laboratory stock colony. Fecal-oral contamination is considered a common route of horizontal transmission of viruses in arthropods.[11,24] In this study, we assessed this mechanism of transmission in *D. pteronyssinus* by demonstrating that at least five of the studied viruses (DerpV1/2/3/5/6) could persistently be transmitted to a virus-free receptor colony (J-1–1–1–1) just by a single inoculation of virus-containing fecal material. This result evidences the infectivity of these viruses, and strongly suggests that, among other possible mechanisms such as vertical transmission, they are transmitted to *D. pteronyssinus* via fecal-oral ingestion. Notably, HDM is known to practice auto-coprophagia presumably as part of their feeding behavior.[2] As expected from our experience with the stock colony that showed optimal performance despite a high viral load, viral infection did not significantly impact population growth in the virus-inoculated colony. Therefore, it appears that *D. pteronysinus* would to some extent tolerate its virome, although the nature of the established host-virus interaction remains unknown. Dicistroviruses, as related to some abundant viruses in our study and showing a similar infection/transmission cycle linked to the digestive system, are known to infect arthropods with high host specificity, although virulence and pathogenicity may vary considerably.[24] It cannot be discarded that these viral infections were beneficial to *D. pteronyssinus*, for instance by playing a protective role against other pathogenic infections as known in other arthropods,[29] or near neutral, by targeting digestive epithelial cells that, as our observations indicate, could already be decaying naturally and being released the gut lumen as free floating cells. Such cells/vesicles have been reported in different mite species, including HDM.[30–32] It is to be noted that, besides population growth, which we used as a general trait of mite performance, no other physiological responses have been addressed in the scope of this study. Thus, it is likely that viral infection produces physiological changes that were not recorded here. Interestingly, all three *D. pteronyssinus* major allergens, Der p 1/2/23, are expressed in the digestive epithelium of the mite and then excreted into fecal pellets,[33–35] similar to the localization of the studied viruses. Therefore, it would be interesting to investigate if these digestive viruses could affect the expression of major allergens, thus the allergenic properties of the mite.

The detection of viral RNA in on other sources besides our laboratory colonies suggests that their occurrence is to be quite widespread in *D. pteronyssinus*. Similar genotypes to most of the viruses under study could also be found in transcriptomes from *D. pteronyssinus* colonies of independent laboratories,[12,22,23] suggesting they are commonly associated to the species independently of the origin of the colony. Moreover, viral presence was not restricted to the laboratory since we could also report viral RNA in wild mite populations retrieved from house dust samples. Following our screening method, two positive samples involving two viral species, DerpV1/2, were detected out of the seven house dust samples with confirmed *D. pteronyssinus* occurrence, both collected in the Galicia region. Note that our study aimed to confirm the possibility of detecting viral RNA in domestic environments rather than assessing their geographical distribution or concentration. The detection of viral RNA in our samples might potentially be underestimated since mite-derived viral titers in house dust are expected to be low and no virus enrichment step was included in our procedure.[11] Additionally, viral RNA from most of the identified viruses could also be detected in pharmaceutical products used to diagnose *D. pteronyssinus* allergy, as well as in the international *D. pteronyssinus* reference extracts used to standardize/certify their production.[36] Such diagnostic products, as well as therapeutic products used for AIT, are formulated essentially from natural extracts obtained from large-scale *D. pteronyssinus* cultures held by Pharma companies or institutions. Thus, the viral fingerprint detected in the end product is to reflect at least part of the viruses that were actually present in the mite colony used to produce the active pharmaceutical ingredient (API). It is to be noted that RT-PCR

detects viral RNA instead of complete virions; thus, their potential viability in these products is unknown. In addition, viral content and integrity in the HDM product may depend on the post-processing of the API in the manufacturing process. The viral profiles of the eight analyzed products differed considerably but, except for the WHO/IUIS standard and the skin-prick testing glycerinate from company 4, all products exhibited RNA of at least one of the picornavirus-like species (ie, DerpV1/2/3). This was also the case for the viruses identified in house dust samples or in *D. pteronyssinus* transcriptomes. Altogether, these findings indicate that a prevalent association may exist between picorna-like viruses and the host species, as recently observed also for other allergenic mites.[12] It should also be stressed that direct sequencing of RT-PCR amplicons for DerpV1/2/3 revealed phylogenetic distinct variants for each of the analyzed samples, also differing from the viral sequences of our in-house stock colony, what discards the possibility of cross-contamination.

Allergy is a complex multifactorial immunological process primarily triggered after exposure to allergens, as elicitors of immunoglobulin E (IgE) response.[37] However, growing attention exists on other molecules produced by the mite, such as proteases[38] or microRNA,[39] which might also be playing a role in allergy through innate immunity IgE-independent pathways. Furthermore, the onset and evolution of allergic diseases can also be driven by environmental factors such as air pollution[40] or the exposure to microbes.[41] In short, features eventually modulating the inflammatory and immunological responses have the potential to interplay in the process of allergy. The same could apply to the mite-derived viruses described herein, although their actual potential to trigger physiological responses in humans is still unknown and needs further experimental scientific evidence. In the case of pathogenic viruses, a clear epidemiologic link has been established between some viral infections and allergy.[14] Mite-derived virions, albeit unlikely replicating in humans, might still share immunoactive components with human pathogenic viruses, such as specific signatures in the structural proteins of the coat or the RNA molecule, which could be similarly recognized by biosensors of the human innate immune system.[42] Their eventual sensing by pattern recognition receptors (PRRs) in epithelial cells could initiate immunological responses that have the potential to interact with allergic sensitization or even allergic symptoms in previously mite-sensitized individuals in the absence viral disease. As a matter of fact, research on human respiratory viruses has shown they share elements of their immunological signal transduction pathways with protease allergen Der p 1, suggesting that both HDM allergy and viral sensing/infection are interconnected.[13] Interestingly, similar findings have been obtained experimentally in the absence of viral infection, but using synthetic agonists of viral nucleic acids.[43] Two aspects demonstrated in our work play in favor of the eventual interaction of mite-derived viruses with human epithelia. First, these viruses could potentially be in the frontline of the natural exposure of human subjects in domestic environments since they are intimately bonded to fecal pellets, which become easily airborne and inhalable,[2] and RNA from some of them could be detected on house dust samples. And, second, since viral RNA has also been detected in pharmaceutical products, HDM allergic patients could also be subjected to a direct contact with mite viruses, or viral components, through different routes.[1] Although the influence of mite viruses on the effects of AIT products remains to be explored, adverse reactions are not expected based on the long track record of safe clinical use of these products. If justified clinically, eventual measures could readily be transferred to the industry to limit the impact of viruses in HDM AIT products. These include the selection of virtually virus-free *D. pteronyssinus* colonies, as shown in our work, or the addition of steps in the manufacturing process to prevent their effects in the end product. The findings and resources generated in this work will be the basis of future investigations to address the clinical impact of HDM viruses on allergy, as well as their possible role in the process of allergen desensitization by AIT, and open the door to similar investigations exploring viromes in other allergenic sources.

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CONFLICT OF INTEREST

JCVQ, CV, FE and SR have no conflict of interest to declare. BNL has received research grants from ALK and GSK. PHC has received a research grant from ALK. None of these grants was related the submitted work.

AUTHOR CONTRIBUTION

JCVQ and PHC designed the experiments. JCVQ carried out bench experiments. PHC performed *in silico* bioinformatic analysis of viral sequences. BNL and SR contributed to RNA sequencing. FE prepared electron microscopy samples and assisted in visualization. CV contributed to sampling of environmental samples and commercial products. All authors participated in the writing of the manuscript and have read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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