First Isolation of a Marseillevirus in the Diptera Syrphidae *Eristalis tenax*

Mondher Boughalmi^a Isabelle Pagnier^a Sarah Aherfi^a Philippe Colson^a
Didier Raoult^{a, b} Bernard La Scola^a

^aURMITE UM63, CNRS 7278, IRD 198, INSERM 1095, Aix-Marseille Université, Marseille, France;

^bSpecial Infectious Agents Unit, King Abdulaziz University, Jeddah, Saudi Arabia

Key Words

Giant virus · Marseillevirus · Marseilleviridae · *Eristalis tenax*

Abstract

Objective: Giant viruses and amoebae are common in freshwater, where they can coexist with various insects. We screened insect larvae to detect giant viruses using a high-throughput method. **Methods:** We analyzed 86 *Eristalis tenax* larvae obtained from stagnant water reservoirs in Tunisia. The larvae were decontaminated and then dissected to remove internal parts for coculture with *Acanthamoeba polyphaga*. Genome sequencing of isolated viruses was performed on a 454 Roche instrument, and comparative genomics were performed. **Results:** One Marseillevirus, named Insectomime virus, was isolated. The genome assembly generated two scaffolds, which were 382,776 and 3,855 bp in length. Among the 477 identified predicted proteins, the best hit for 435 of the identified proteins was a Marseillevirus or Lausannevirus protein. Tunisvirus was the most closely related to Insectomime, with 446 orthologs. One Insectomime protein shared with Lausannevirus and Tunisvirus showed the highest similarity with a protein from an aphid. **Conclusion:** The isolation of a Marseillevirus from an insect expands the diversity of environments in which giant viruses have been isolated. The coexistence of larvae and giant viruses in stagnant water may explain the presence of the giant virus

in the larva internal structures. This study illustrates the putative role of amoeba in lateral gene transfer not only between the organisms it phagocytoses, but also between organisms living in the same environment.

© 2013 S. Karger AG, Basel

Introduction

The order Megavirales is composed of unusual viruses in terms of the size of their viral particles and the size [1, 2] and contents of their genomes; these characteristics of this order differ considerably from the canonical definition of viruses [3]. These viruses are found in different ecological niches [4, 5], reflecting their ability to adapt to different environmental conditions and physico-chemical stress, such as hypersalinity [6].

Of the amoeba-associated giant viruses, Megavirales are represented by the families Mimiviridae [1] and Marseilleviridae [7]. The founder of these two families is Marseillevirus [8]. Amoebae occupy several environmental ecological niches and are commonly found in freshwater, where they can coexist with the larvae of several insect species. Amoebae can be found ubiquitously, under various environmental conditions, such as freshwater, marine water, soil, vertebrates, aerial parts of plants and animals [9]. Some species have been isolated from extreme

environments, such as hypersaline [10] or hyperthermic [11] conditions. *Acanthamoeba* have also been described colonizing insects [12]. For some other insect species, such as the syrphid fly *Eristalis tenax* (Diptera: Syrphidae), eggs normally remain on the surface of water. Larval development is represented by three stages of metamorphosis, larvae, pupas and imago, which have all been demonstrated to be potential vectors for microbial pathogens [13]. *E. tenax* is also important in some cases of intestinal, nasal, genital and urinary myiasis [14–17]. The three stages of metamorphosis occur in aquatic environments, such as stagnant water, and are in contact with the same environment as amoebae. The immature form of *E. tenax* is referred to as a rat-tailed maggot. The adult form is commonly known as the drone fly and is part of the population of pollinators. The decline in the numbers of these insects is continuing and has significant environmental and economic consequences, mainly because of their great agronomic impact [18]. Several studies have explained that this decline is due to multiple factors, including exposure to pesticides, land use changes, decreasing genetic diversity and climate change [19, 20]. The increasingly frequent presence of several types of parasites in pollinator insects may also be important. Parasites are important in exerting selection pressure for most organisms, including insects. Some insect parasites are highly virulent and cause diseases with evident symptoms, such as the fungi *Metarhizium* spp. and *Ascospaera* spp., which infect several species of arthropods, including termites and bees [21–23]. Other parasites have less obvious pathological consequences, such as the intracellular bacteria *Wolbachia* spp., which is widespread in insects. *Wolbachia* spp. has been shown to have a major impact on the physical condition of the host by manipulating the sex ratio or by adversely affecting insect survival [24, 25]. In addition to fungi and bacteria, some viruses can also be identified as parasites of arthropods. For example, members of the families Dicistroviridae and Iflaviridae can infect honey bees, which are pollinator insects [26]. All of these parasites may be responsible for the decrease in the number of pollinator populations and are capable of the interspecies transmission, infection or vectorization of infectious diseases. A recent study of the transcriptome of the insect *Lymantria dispar* [27] revealed the presence of gene sequences belonging to Mimivirus, which is classified in lineage A of the amoebae-associated mimiviruses within the order Megavirales. This observation raises the question of whether there is a relationship between insect species and giant viruses. We hypothesize that there may be multiple lateral transfers of genes, as was suggested by

Fig. 1. Morphological identification of larvae: maggots (a) and pupae (b).

Table 1. Sampling sites and characteristics of the samples used for the inoculation of the cocultures

Samples (n = 86)	Maggots (n = 46)	Pupae (n = 40)
Stagnant water tank 1	19	23
Stagnant water tank 2	27	17

Bertelli et al. [28]. In our study, we screened several insect larvae to detect the presence of giant viruses using a high throughput detection method. We isolated from a larva of the species *E. tenax* a new giant virus, which can be classified in the family Marseilleviridae.

Materials and Methods

Sample Preparation

Insect larvae were taken from two distinct reservoirs of stagnant water (table 1). There was no renewing of the water, and the ambient temperature was 12° at the time of sampling, and the wa-

Fig. 2. Schematic description of the method used to isolate giant viruses from insect larvae.

ter temperature was 17°. Those reservoirs were located in the city center of Tunis, Tunisia, which has a Mediterranean climate. Eighty-six larvae of the fly species *E. tenax* were taken from the backwaters of these reservoirs. To prevent pupation during transportation, wet bottles were used to transport the larvae in high humidity. Identification of the larvae and adult insects was performed morphologically, using the characteristic rat-tailed aspect of the maggots (fig. 1). *E. tenax* larvae were killed with a 96% ethanol wash for 20 min, which also decontaminated their surfaces. The larvae were then washed in a washing solution that consisted of sterile Page's amoebal saline (PAS) buffer, and washing suspensions were kept for further investigation. The larvae were dissected as follows: they were wrapped in paraffin and an incision was made on the side of the abdomen with a sterile scalpel, the digestive tract and internal organs were then harvested under sterile conditions and separated from the rest of the larval body. The digestive tract and internal organs (DT+IO) were crushed mechanically in a sterile mortar with 3 ml of PAS buffer, and the suspension was homogenized. The same treatment was performed for the rest of the larvae bodies (LB). A solution consisting of a mixture of 4 antibiotics [10 µl of ciprofloxacin at 4 mg/ml (Pan-

pharma, Clairay, France), 10 µl of vancomycin at 4 mg/ml (Mylan, Saint-Priest, France), 10 µl of colimycin at 500 IU/ml (Sanofi Aventis, Paris, France), 10 µl of rifampicin at 4 mg/ml (Sanofi Aventis)] and 1 antifungal agent [10 µl of Fungizone at 100 mg/ml (Bristol Myers Squibb, Rueil-Malmaison, France)] was added to the suspensions to eliminate bacterial and fungal contamination. The homogenized suspensions of the DT+IO and LB were washed with 5 successive centrifugations at 15,000 rpm for 30 min and then suspended in PAS buffer to remove traces of the antimicrobial solution. The pellets were resuspended in 500 µl of PAS buffer. The efficiency of the antimicrobial treatment was assessed by inoculating 50 µl of the suspension on BCYE agar (Oxoid, Dardilly, France) and Columbia sheep blood agar (BioMérieux SA, Marcy L'Etoile, France). The agar plates were incubated for at least 5 days at 32°.

Giant Virus Research

One hundred microliters of the final suspensions of the DT+IO and LB were used to study the giant viruses with a high throughput isolation system, as described elsewhere [6], using the amoeba species *Acanthamoeba polyphaga*, strain Linc-AP1. Briefly, a primary

Fig. 3. Morphology of the Insectomime virus. **a** Hemacolor staining of *A. polyphaga* infected by Insectomime virus. VF = Virus factories. **b** TEM image of the isolated virus showing the typical Marseilleviridae particle morphology of Insectomime.

Table 2. Sequences of the primers used for the preliminary molecular identification of Insectomime

Targeted gene	Primer name	Primer sequence	Temp. (°)
Major capsid protein	T19capF	5'-ATCTCCGGAACGATTCACAG-3'	60
	T19capR	5'-TAACTGCTGCTTCCGGTTTT-3'	58
D6 helicase	T19D6/D11F	5'-AGTTACCCAACCACAAGAAGA-3'	58
	T19D6/D11R	5'-CAGAAGGACTAACAAAAGAACCA-3'	60

culture was made by inoculating 100 µl of the sample onto an amoebal monolayer in 12-well microplates without antibiotics. A second enrichment step was performed by subculturing the primary cultures for 3 days on a fresh amoebal monolayer with the addition of the antibiotic cocktail described above that was used for sample decontamination. Finally, these subcultures were inoculated onto agar plates that were coated with an amoebal monolayer. The presence of viruses was assessed through the development of a lysis plaque that was visible to the naked eye. To confirm the presence of giant viruses outside of the larvae, the first washing solutions were systematically centrifuged at 15,000 rpm for 30 min, and the pellet was suspended in 500 µl of sterile PAS. Following the first resuspension the pellet was washed five times with successive centrifugations and resuspensions, as described above, to remove traces of ethanol. The final pellet was resuspended in 500 µl of sterile PAS and tested for the presence of giant viruses using the same high throughput isolation method. When a lysis plaque was observed on the high-throughput agar plate, agar was cut out of the plaque, divided into small pieces, resuspended in PAS buffer and filtered with a 1.2-µm-pore filter. The filtrate was then re-inoculated onto a fresh amoebal monolayer in a 12-well microplate. The presence of the giant virus in the culture was first assessed by preparing slides with 100 µl of culture through cytocentrifugation and Hemacolor staining to determine if the virus was being produced in the infected amoebae. After the culture was lysed, the supernatant was negatively stained with a 3% ammonium molybdate solution and visualized with an electron microscope. The observed virus was then assessed for a preliminary morphological classification of the family Mimiviridae or Marseilleviridae (fig. 2).

Molecular Detection and Preliminary Identification of the Isolated Giant Viruses

For the larvae that contained a giant virus, DNA was extracted from 200 µl of the DT+IO suspension, 200 µl of the LB suspension and 200 µl of the corresponding wash solution. The DNA extraction was performed using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. DNA was also extracted from a suspension of the isolated virus for preliminary identification following morphological presumptive characteristics. A PCR reaction was performed, as described elsewhere [6], using primer pairs specific for Marseillevirus (table 2). For positive samples, the same primer pair was used for sequencing in an ABI PRISM 3130XL DNA sequencer (Applied Biosystems). The sequences were assembled and then analyzed with ChromasPro software (Technelysium Pty Ltd.). The assembled sequences were compared with sequences available in the GenBank database using BLAST software (<http://blast.ncbi.nlm.nih.gov/>).

Whole Genome Sequencing

The Insectomime genome was sequenced on a 454 Roche GS20 instrument, as previously described [8]. The reads obtained were assembled de novo with Newbler Assembly software [29]. Protein encoding genes were predicted using the GeneMarkS tool [30]. Orthologous genes were searched for using the Proteinortho program [31] and were considered as the best reciprocal hits using a threshold of an *e* value $<1e-3$, an amino acid identity of $>30\%$ and a coverage of $>70\%$ on both the query and target sequences. To detect all the orthologs shared with other

Fig. 4. Phylogenetic reconstruction based on a concatenated alignment of the D6-D11 helicase, the major capsid protein, and the DNA polymerase B family from the members of the family Marseilleviridae. *A. polyphaga mimivirus*, another member of Megavirales, was used as an outgroup using the maximum likelihood method with Mega 5 software (<http://www.megasoftware.net/>). The analysis involved a total of 1,580 positions in the final dataset. The probabilities are shown near the branches as percentages and are used as confidence values for the tree branches. The scale bar represents the

number of estimated changes per position for a unit of branch length. D6-D11 helicase: Marseillevirus: YP_003406944.1, Lausannevirus: YP_004347182.1; major capsid protein: Marseillevirus: YP_003407071.1, Lausannevirus: YP_004347316.1; DNA polymerase B family: Cannes 8: accession No. JF979175.1, Tunisvirus: GenBank submission grp 4154826, Insectomime: GenBank accession submission ID 1632859, Fontaine Saint-Charles: GenBank accession submission ID 1632860 [6].

marseilleviruses (Marseillevirus [8], Lausannevirus [32] and Tunisvirus fontaine 2 [6]), we performed a non-reciprocal BLASTp search [33, 34], using the thresholds $1e-4$ for the *e* value and at least 25% for both the query and target coverage. Transfer RNA was searched for with the tRNAscan-SE web server [35].

Phylogeny Reconstruction

To construct a phylogenetic tree, proteic sequences were aligned using MUSCLE software [36], the informational positions were selected using Gblocks [37] and the maximum likelihood phylogeny was performed using MEGA5 software [38]. The phylogeny reconstruction, involving the marseilleviruses and *A. polyphaga mimivirus* as an outgroup, was performed after a concatenation of the selected blocks of the D6-D11 helicase, the major capsid protein, and the DNA polymerase B sequences.

Results

Isolation and Preliminary Morphological and Molecular Identification

The use of a high-throughput isolation system allowed for the isolation of a single virus from 86 *E. tenax* larvae. The virus was isolated from the internal organs and digestive tract of a maggot from reservoir tank 1, but not from the surface of the larvae or the washing solution, either by culture or by PCR. The surface of the viral lysis plaque was 49 mm² after an incubation time of 12 h. Hemacolor

staining of the amoebal coculture showed virus factory accumulation 12 h after infection (fig. 3a). Measurement with an electron microscope showed an overall viral particle diameter of 225 nm, with a typical icosahedral structure of giant viruses (fig. 3b), suggesting that it was a virus of the family Marseilleviridae. The results of the PCR and sequencing analysis confirmed this classification of the family Marseilleviridae. The virus was called Insectomime.

Whole Genome Sequencing of Insectomime

Insectomime genome sequencing generated 272,503 reads. Assembly of the reads resulted in 9 large contigs with a mean size of 42,306 bp. The contigs assembled themselves in scaffolds of 382,776 and 3,855 bp. The Insectomime draft genome (GenBank submission ID 1632859) is a double-stranded DNA molecule composed of approximately 386,000 bp, with a guanine-cytosine content of 42.7%. Phylogeny based on a concatenated alignment of the D6-D11 helicase, the major capsid protein and the DNA polymerase B family sequences showed the position of this virus among previously isolated Marseillevirus, Lausannevirus and Tunisvirus, which is its closest neighbor (fig. 4). A tree constructed based on the family B DNA polymerase exhibited the same topology. Comparative analyses using BLASTp searches of the Insectomime genome and ge-

Fig. 5. Dot plot between orthologous proteins of Insectomime and Marseillevirus (a), Lausannevirus (b) or Tunisvirus fontaine 2 (c).

nomes of other marseilleviruses showed substantial levels of similarity and collinearity, particularly at the ends of the genome (fig. 5). A total of 477 predicted proteins were tentatively identified in the Insectomime genome, with their sizes varying from 46 to 1,643 amino acids. Of these proteins, 435 were found to be best hits for either a Marseillevirus or a Lausannevirus protein. Insectomime shares 311 and 357 orthologs with Marseillevirus and Lausannevirus, including 260 and 300 bona fide orthologous genes (involved in reciprocal best hits with a sequence coverage >70%), respectively. Of the analyzed marseilleviruses, Tunisvirus appeared to be the most closely related to Insectomime. Insectomime shares 446 orthologs with Tunisvirus, including 345 reciprocal best hits (fig. 5c). Otherwise, 329 Insectomime proteins were annotated as hypothetical proteins, including 293 found either in Marseillevirus or Lausannevirus, two proteins from *A. castellanii* strain Neff (ACA1_085270) and *A. polyphaga moumouvirus* (AGC02396.1), and 34 ORFs, with sizes ranging from 103 to 244 amino acids. Several orthologs to serine/protein kinases, proteins containing bacterial-like membrane occupation and recognition nexus repeat domains, histone-like proteins and endonucleases (especially restriction endonucleases and Vsr/MutH/archaeal HJR family endonucleases) were identified, as was first determined for Marseillevirus [8, 32]. Apart from proteins of the marseilleviruses, best BLASTp hits for eight Insectomime proteins were found in *A. castellanii*, *A. polyphaga moumouvirus*, an amoebae Mimivirus of the lineage B, a flagellate (*Cercomonas* spp.), two opisthokonts (*Capsaspora owczarzaki*, *Hydra*

magnipapillata), a bacterium (*Cellulophaga algicola*), a phage of a free-living nitrogen-fixing bacterium (*Azospirillum* phage Cd), and in an aphid (*Acyrtosiphon pisum*). In the aphid case, the Insectomime protein, orf298, is an ankyrin repeat-containing protein that is also found in Lausannevirus (YP_004347163.1) and Tunisvirus (Tunisvirus_orf78). The Insectomime genome encodes 6 ankyrin repeat-containing proteins, which is equivalent to what is found in the Tunisvirus genome but greater than what is found in the genomes of the other marseilleviruses. Eight of the twenty best hits found by a BLASTp search for Insectomime orf298 against the NCBI GenBank non-redundant protein sequence database matched to putative ankyrin repeat-containing proteins from *Acyrtosiphon pisum*. For the top hit (XP_001949966.2), the *e* value was $7e-9$, the amino acid identity was 26% and the query (Insectomime orf298) and target coverage were 80 and 66%, respectively. The twelve other best target sequences were proteins from Lausannevirus, *A. castellanii*, three chlorophyta (*Ostreococcus lucimarinus*, *Bathycoccus prasinus* and *Micromonas pusilla*), and a heterokontophyta (*Phytophthora infestans*) (fig. 6).

Discussion

We found one giant virus from the recently described family Marseilleviridae [7] in the 86 larvae studied. The virus is phylogenetically distinct from other previously described marseilleviruses, including Marseillevirus [8],

Fig. 6. Phylogenetic reconstruction based on an alignment of an ankyrin repeat-containing protein from Insectomime and orthologous proteins, using the maximum likelihood method with Mega 5 software (<http://www.megasoftware.net/>). The probabilities are

shown near the branches as percentages and are used as confidence values for the tree branches. The scale bar represents the number of estimated changes per position for a unit of branch length.

Lausannevirus [32], Cannes 8 [unpubl. data] (GenBank submission ID 1632635). Tunisvirus [unpubl. data] (GenBank submission ID 4154826) [6] is the most closely related virus to the new virus. This new virus was found only in the internal organs of the insect, including the digestive tract, and no virus could be found in the surface or in the suspension used to wash the larvae before the experiment. The species *E. tenax* (Diptera, Brachycera, Muscomorpha, Syrphidae, *E. tenax*) is an insect with a complex metamorphosis process. The eggs are typically laid on the surface of water and the larvae proceed through three stages of development. Metamorphosis always occurs in an aquatic environment that is rich in organic matter, such as stagnant water reservoirs. As was previously described for amoebae [39], the species *E. tenax* is also an important vector for several bacterial species, such as *Mycobacterium* spp. [8], which is responsible for a disease in pigs. To our knowledge, this is the first study demonstrating that a giant virus has been isolated from this species in particular and from an insect in general. The coexistence of larvae and giant viruses in stagnant water may explain the presence of a giant virus in the internal tracts of the larvae. Giant viruses are most often found in aquatic environments [5, 6], and the possibility that the virus was ingested by the insect larva might explain its presence in only the internal organs of the animal and not

on the surface. Otta et al. [12] described that amoeba can colonize some insect species; this raises a question about the origin of the virus presence in the insect. The virus could have been directly ingested, or it could have entered the insect through the ingestion of an infected amoeba. The capacity of amoebae to act as ‘Trojan horses’ has already been described [40] and could explain the presence of the virus in the insect larva. Our findings illustrate the possible role of giant viruses in the transfer of genes between microorganisms, through cocolonization with other microorganisms of the same eukaryotic hosts [41–43]. This study also suggests the multilateral transfer of genes between different species of the same ecological niche, such as insect larvae and amoebae in stagnant water. In the viral genome, among the eight Insectomime proteins having as their best BLASTp hit an organism other than Marseillevirus or Lausannevirus, five proteins belonging to eukaryotes were found. One Insectomime protein harbored a high similarity (e value: $7e-9$; coverage: 80% for the viral protein, 66% for the insect protein; amino-acid identity: 26%; similarity: 40%) to a protein from an insect from the family Aphididae (*Acyrtosiphon pisum*). Ankyrin repeat domain-containing proteins mediate protein-protein interactions and have been described to be involved in the lateral transfer of genes between eukaryotes and bacteria [44]. In ecological niches, such as reser-

voirs of stagnant water, amoebae are presented as a ‘melting pot’, a giant hub for the exchange of genes between amoeba-resistant bacteria, virophages and giant viruses [8]. The data reported here suggest that ankryrin repeat proteins may have undergone a lateral gene transfer between giant viruses in amoebae and insects, especially those having at least an aquatic phase in their developmental cycle and a greater opportunity for contact with amoebae. Future studies can address the effects of the virus on the development and survival of the larvae to eval-

uate the impact of this infection on the pollinator populations and to establish the role of emerging viruses in the decline of some nearly extinct populations.

Disclosure Statement

The authors declare that there is no potential conflict of interest or financial disclosure.

References

- 1 Raoult D, Audic S, Robert C, Abergel C, Renesto P, Ogata H, La Scola B, Suzan M, Claverie JM: The 1.2-megabase genome sequence of Mimivirus. *Science* 2004;306:1344–1350.
- 2 La Scola B, Audic S, Robert C, Jungang L, de Lamballerie X, Drancourt M, Birtles R, Claverie JM, Raoult D: A giant virus in amoebae. *Science* 2003;299:2033.
- 3 Colson P, de Lamballerie X, Fournous G, Raoult D: Reclassification of giant viruses composing a fourth domain of life in the new order Megavirales. *Intervirology* 2012;55:321–332.
- 4 Yamada T: Giant viruses in the environment: their origins and evolution. *Curr Opin Virol* 2011;1:58–62.
- 5 La Scola B, Campocasso A, N'Dong R, Fournous G, Barrassi L, Flaudrops C, Raoult D: Tentative characterization of new environmental giant viruses by MALDI-TOF mass spectrometry. *Intervirology* 2010;53:344–353.
- 6 Boughalmi M, Saadi H, Pagnier I, Colson P, Fournous G, Raoult D, La Scola B: High-throughput isolation of giant viruses of the Mimiviridae and Marseilleviridae families in the Tunisian environment. *Environ Microbiol* 2013;15:2000–2007.
- 7 Colson P, Pagnier I, Yoosuf N, Fournous G, La Scola B, Raoult D: ‘Marseilleviridae’, a new family of giant viruses infecting amoebae. *Arch Virol* 2013;158:915–920.
- 8 Boyer M, Yutin N, Pagnier I, Barrassi L, Fournous G, Espinosa L, Robert C, Azza S, Sun S, Rossmann MG, Suzan-Monti M, La Scola B, Koonin EV, Raoult D: Giant Marseillevirus highlights the role of amoebae as a melting pot in emergence of chimeric microorganisms. *Proc Natl Acad Sci USA* 2009;106:21848–21853.
- 9 Rodriguez-Zaragoza S: Ecology of free-living amoeba. *Crit Rev Microbiol* 1994;20:225–241.
- 10 Park JS, Simpson AG, Brown S, Cho BC: Ultrastructure and molecular phylogeny of two heterolobosean amoebae, *Euplaesiobystra hypersalinica* gen. et sp. nov. and *Tulamoeba peronaphora* gen. et sp. nov., isolated from an extremely hypersaline habitat. *Protist* 2009;160:265–283.
- 11 De Jonckheere JF, Baumgartner M, Oppendoes FR, Stetter KO: *Marinamoeba thermophila*, a new marine heterolobosean amoeba growing at 50 degrees C. *Eur J Protistol* 2009;45:231–236.
- 12 Otta DA, Rott MB, Carlesso AM, da Silva OS: Prevalence of *Acanthamoeba* spp. (Sarcomastigophora: Acanthamoebidae) in wild populations of *Aedes aegypti* (Diptera: Culicidae). *Parasitol Res* 2009;111:2017–2022.
- 13 Fischer OA, Mátlová L, Dvorská L, Svástová P, Bartos M, Weston RT, Pavlík I: Various stages in the life cycle of syrphid flies (*Eristalis tenax*; Diptera: Syrphidae) as potential mechanical vectors of pathogens causing mycobacterial infections in pig herds. *Folia Microbiol* 2006;51:147–153.
- 14 Raffray L, Malvy D: Accidental intestinal myiasis caused by *Eristalis tenax* in France. *Travel Med Infect Dis* 2013, E-pub ahead of print.
- 15 Salimi M, Edalat H, Jourabchi A, Oshaghi M: First report of human nasal myiasis caused by *Eristalis tenax* in Iran (Diptera: Syrphidae). *Iran J Arthropod Borne Dis* 2010;4:77–80.
- 16 González M, Comte MG, Monárdez PJ, Díaz de Valdés LM, Matamala CI: Accidental genital myiasis by *Eristalis tenax*. *Rev Chilena Infectol* 2009;26:270–272.
- 17 Mumcuoglu I, Akarsu GA, Balaban N, Keles I: *Eristalis tenax* as a cause of urinary myiasis. *Scand J Infect Dis* 2005;37:942–943.
- 18 Golding YC, Ennos AR, Edmunds M: Similarity in flight behaviour between the honeybee *Apis mellifera* (Hymenoptera: apidae) and its presumed mimic, the dronefly *Eristalis tenax* (Diptera: syrphidae). *J Exp Biol* 2001;204:139–145.
- 19 Ratnieks FL, Carreck NL: Ecology: clarity on honey bee collapse? *Science* 2010;8:152–153.
- 20 Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE: Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* 2010;25:345–353.
- 21 Bérénos C, Schmid-Hempel P, Wegner KM: Evolution of host resistance and trade-offs between virulence and transmission potential in an obligately killing parasite. *J Evol Biol* 2009;22:2049–2056.
- 22 Aronstein KA, Murray KD: Chalkbrood disease in honey bees. *J Invertebr Pathol* 2010;103:S20–S29.
- 23 Evison SE, Roberts KE, Laurenson L, Pietravalle S, Hui J, Biesmeijer JC, Smith JE, Budge G, Hughes WO: Pervasiveness of parasites in pollinators. *PLoS One* 2012;7:e3064.
- 24 Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH: How many species are infected with Wolbachia? A statistical analysis of current data. *FEMS Microbiol Lett* 2008;281:215–220.
- 25 Werren J: Biology of Wolbachia. *Annu Rev Entomol* 1997;42:587–609.
- 26 Evans JD, Schwarz RS: Bees brought to their knees: microbes affecting honey bee health. *Trends Microbiol* 2011;19:614–620.
- 27 Sparks ME, Gundersen-Rindal D: The *Lymantria dispar* IPLB-Ld652Y cell line transcriptome comprises diverse virus-associated transcripts. *Viruses* 2011;3:2339–2350.
- 28 Bertelli C, Greub G: Lateral gene exchanges shape the genomes of amoeba-resisting microorganisms. *Front Cell Infect Microbiol* 2012;2:110.
- 29 Margulies M, Egholm M, Altman WE, et al: Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005;437:376–380.
- 30 Besemer J, Borodovsky M: GeneMark: Web software for gene finding in prokaryotes, eukaryotes and viruses. *Nucleic Acids Res* 2005;33:W451–W454.

- 31 Lechner M, Findeiss S, Steiner L, Marz M, Stadler PF, Prohaska SJ: Proteinortho: detection of (co-)orthologs in large-scale analysis. *BMC Bioinformatics* 2011;12:124.
- 32 Thomas V, Bertelli C, Collyn F, Casson N, Telenti A, Goesmann A, Croxatto A, Greub G: Lausannevirus, a giant amoebal virus encoding histone doublets. *Environ Microbiol* 2011;13:1454–1466.
- 33 Desnues C, La Scola B, Yutin N, Fournous G, Robert C, Azza S, Jardot P, Monteil S, Campocasso A, Koonin EV, Raoult D: Provirophages and transpovirons as the diverse mobilome of giant viruses. *Proc Natl Acad Sci USA* 2012;109:18078–18083.
- 34 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. *J Mol Biol* 1990;215:403–410.
- 35 Schattner P, Brooks AN, Lowe TM: The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Res* 2005;33:W686–W689.
- 36 Edgar RC: Muscle: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797.
- 37 Castresana J: Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 2000;17:540–552.
- 38 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;28:2731–2739.
- 39 Salah IB, Ghigo E, Drancourt M: Free-living amoebae, a training field for macrophage resistance of mycobacteria. *Clin Microbiol Infect* 2009;15:894–905.
- 40 Greub G, Raoult D: Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev* 2004;17:413–433.
- 41 Raoult D, Boyer M: Amoebae as genitors and reservoirs of giant viruses. *Intervirology* 2010;53:321–329.
- 42 Thomas V, Greub G: Amoebae/amoebal symbionts genetic transfers: lessons from giant virus neighbours. *Intervirology* 2010;53:254–267.
- 43 Moliner C, Fournier PE, Raoult D: Genome analysis of microorganisms living in amoebae reveals a melting pot of evolution. *FEMS Microbiol Rev* 2010;34:281–294.
- 44 Al-Khodori S, Price CT, Kalia A, Abu Kwaik Y: Functional diversity of ankyrin repeats in microbial proteins. *Trends Microbiol* 2010;18:132–139.