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Adventitious viruses in insect cell lines used for recombinant protein expression

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Abstract

Insect cells are widely used for recombinant protein expression, typically as hosts for recombinant baculovirus vectors, but also for plasmid-mediated transient transfection or stable genetic transformation. Insect cells are used to express proteins for research, as well as to manufacture biologicals for human and veterinary medicine. Recently, several insect cell lines used for recombinant protein expression were found to be persistently infected with adventitious viruses. This has raised questions about how these infections might affect research performed using those cell lines. Furthermore, these findings raised serious concerns about the safety of biologicals produced using those cell lines. In response, new insect cell lines lacking adventitious viruses have been isolated for use as improved research tools and safer biological manufacturing platforms. Here, we review the scientific and patent literature on adventitious viruses found in insect cell lines, affected cell lines, and new virus-free cell lines.

Keywords

Adventitious viruses; Baculovirus insect cell system; Insect cell lines; Sf-rhabdovirus; *Spodoptera frugiperda* cells

1. Introduction

The first immortalized insect cell lines were established in the 1960s [1]. Since then, hundreds of insect cell lines have been established from insects in many different Orders, with 940 different cell lines listed in the ExPASy Cellosaurus database at the time of this writing (<http://web.expasy.org/cellosaurus>). Several of these cell lines are routinely used to express recombinant proteins for basic research. In addition, some insect cell lines are now used to manufacture biologicals approved for use in human or veterinary medicine (see [2] for a recent list). The insect cell lines most commonly used for recombinant protein

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expression are derived from the Orders Lepidoptera (moths and butterflies) and Diptera (flies).

Lepidopteran cell lines are typically used as hosts for recombinant baculoviruses encoding the protein(s) of interest (recently reviewed by [3]). Alternatively, they can express recombinant proteins following transient transfection or stable genetic transformation with insect-specific expression plasmids encoding the protein(s) of interest (reviewed by [4, 5]). Lepidopteran cell lines used for recombinant protein expression include lines derived from *Trichoplusia ni* (Tn), such as TN-368 [6], BTI-Tn5B1-4 (commercialized as High Five™ [7]), and Tni PRO (Expression Systems, LLC), lines derived from *Spodoptera frugiperda* (Sf), such as IPLB-Sf21AE (Sf21; [8]), Sf9 [9], and Sf900+ (commercialized as *expresSF+*; [10]), and a few lines derived from *Bombyx mori* (Bm), such as Bm-N [11].

Dipteran insect cell lines are typically used for plasmid-mediated recombinant protein expression [4, 5]. Like mammalian cells, they are not susceptible to baculovirus infection, but can be transduced with baculovirus vectors [12, 13]. The dipteran cell lines most commonly used for recombinant protein expression are S2 and S2R+ both derived from *Drosophila melanogaster* (Dm) [14, 15].

A problem with insect cell-based recombinant protein expression is all the relevant cell lines are persistently infected with various adventitious viruses (see below). The absence of obvious cytopathic effects (CPEs), such as syncytia formation, nuclear hypertrophy, apoptosis, or inclusion body formation allowed these infections to go undetected for decades. The specific questions raised by the presence of adventitious viral contaminants in these cell lines focus on the validity of conclusions obtained using these lines for basic research and the potential biosafety hazards associated with their use as substrates for biologicals manufacturing.

In this review, we have compiled information available on persistent viral infections in insect cell lines used to produce recombinant proteins, which is scattered throughout the scientific and patent literature. In addition, we discuss new insect cell lines that are not contaminated with adventitious viruses. Finally, we briefly discuss measures to prevent contaminating virus-free cell lines.

2. Sf-rhabdovirus (Mononegavirales; Rhabdoviridae)

2.1. Discovery and characteristics

Sf-rhabdovirus was independently discovered in various Sf cell lines at about the same time by three different groups. One group at the FDA's Center for Biologicals Evaluation and Research (CBER) discovered Sf-rhabdovirus in Sf9 and Sf21 cells by using a combination of degenerate PCR and massively parallel sequencing [16]. A separate group at Takeda Vaccines discovered Sf-rhabdovirus in a noroviral vaccine candidate produced by Sf9 cells infected with a recombinant baculovirus encoding a norovirus capsid protein. They found these cells produced not only the expected norovirus-like and baculovirus particles, but also a distinct, unexpected type of particles. These were subsequently identified as Sf-rhabdovirus by sequencing cDNA clones derived from particle RNA. A third group at the

Scripps Research Institute found Sf-rhabdovirus sequences in virus-like particles produced by Sf cells infected with a recombinant baculovirus encoding the *Nudaurelia capensis* omega virus (NωV) capsid protein [17]. This group did not investigate further, but noted some assembled contigs “bore weak protein homology to other Rhabdoviridae and so may reflect a low-level infection with an unknown insect virus.” Our follow-up indicated these assembled contigs were in fact derived from Sf-rhabdovirus.

Sf-rhabdovirus is a typical rhabdovirus with a single stranded, negative sense genome of approximately 13.5 kb encoding canonical rhabdoviral nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), and RNA-dependent RNA polymerase (L), in this order. Some isolates encode an additional ORF, designated “X”, which is located between the G and L ORFs and encodes a putative viroporin [18]. The X gene can be lost upon continuous passage of infected Sf cells and is not required for Sf-rhabdovirus infectivity *in vitro* [87]. In Sf9 cells, the Sf-rhabdovirus genome copy number ranges from 1.5 to 3.3×10^3 RNA molecules/cell. In addition, the virus is shed into the supernatant and Sf9 cell conditioned medium contains $0.9 - 2.9 \times 10^7$ Sf-rhabdovirus RNA genomes / mL [19].

2.1.1. Sf-rhabdovirus is likely derived from Sf caterpillars—Endogenous viral elements (EVE's) are virus-derived DNA fragments embedded in germline chromosomes. Sf cells contain 4 EVE's that are closely related to Sf-rhabdovirus, suggesting this virus was historically associated with Sf populations [2]. To determine if Sf-rhabdovirus is still associated with fall armyworm populations, we searched the transcriptome of Sf adults reared at Frontier Agricultural Sciences in Newark, DE, USA [20] for Sf-rhabdovirus sequences. We found sequences covering the entire Sf-rhabdovirus genome, including the X gene. This ‘wild-type’ Sf-rhabdovirus is 98.0% identical across 13238 nucleotides to the reference sequence (GenBank Acc. No. KF947078) reported by Ma *et al.* in 2014 (C. Geisler, unpublished). This new finding supports the ideas that (i) Sf-rhabdovirus can persistently infect Sf populations, and (ii) persistent infection of Sf caterpillars was the ultimate source of the infections recently detected in Sf cell lines.

2.2. Contaminated, susceptible, and refractory cell lines

The surprising discovery of Sf-rhabdovirus in cell lines used for biologicals manufacturing and basic research has stimulated interest in determining if other cell types are contaminated with and/or susceptible to Sf-rhabdovirus infection. The main experimental approach used to test for Sf-rhabdovirus contamination has been by probing for Sf-rhabdovirus RNA using RT-PCR. The approach used to assess susceptibility to Sf-rhabdovirus infection has been to perform infectivity assays, which involve adding conditioned cell medium containing infectious virus to cell cultures, allowing time for viral adsorption, removing the inoculum, and then serially passaging the cells and periodically assaying cell extracts and/or cell-free media for Sf-rhabdovirus RNA by RT-PCR. If a virus-specific PCR signal is observed and its intensity increases with time after inoculation, this is taken as evidence the virus has infected and replicated in the cells and they are susceptible. If the intensity of the virus-specific PCR signal decreases and eventually disappears, this is taken as evidence the cells are not susceptible to infection.

Generally, studies on Sf-rhabdovirus contamination and susceptibility revealed some lepidopteran insect cell lines in addition to those derived from Sf are contaminated with or susceptible to infection with Sf-rhabdovirus. It should also be noted that Sf-rhabdovirus has not been reported to cause CPEs in any cell line. Furthermore, as of this writing, no mammalian cell lines have been found to be contaminated with or susceptible to infection with Sf-rhabdovirus [16, 19, 87].

2.2.1. Contaminated insect cell lines—Various groups have now shown Sf-rhabdovirus contaminates Sf21 [8], Sf9L5814 [10], and Sf9 [9] cells [16, 17, 19, 21, 22]. Since Sf9L5814 was derived from Sf9, and Sf9 was derived from Sf21, the presence of Sf-rhabdovirus in these daughter and granddaughter cell lines most likely reflects a persistent infection inherited from Sf21. In turn, this most likely reflects persistent infection of the insects from which this cell line was derived, as noted above.

Beyond Sf, it has been reported BCIRL-HS-AM1, a cell line derived from *Heliothis subflexa* [23], is contaminated with Sf-rhabdovirus [19]. In addition, *Bombyx mori* Bm-N cells [11] are contaminated with Sf-rhabdovirus based on the identification of a Bm-N EST (Genbank Acc. No. AK377209.1) identical to a portion of the Sf-rhabdovirus genome (C. Geisler, unpublished). We have extended this result by assaying low passage Bm-N cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) using RT-PCR, which produced strong signals for the entire Sf-rhabdovirus genome (*A. Maghodia*, pers. comm.). A search of the *Spodoptera litura* SL221 cell line transcriptome [24] indicated this cell line is also contaminated with Sf-rhabdovirus (C. Geisler, unpublished).

2.2.2. Susceptible insect cell lines—Infectivity assays have demonstrated Sf-RVN (Sf-rhabdovirus negative), which is an Sf cell line that is not persistently infected with Sf-rhabdovirus [21], is susceptible to experimental infection with Sf-rhabdovirus [87]. Similarly, BCIRL/AMCY-SE-E1, -E4, and -E5, which are three cell lines derived from *Spodoptera exigua* [25], were shown to be susceptible to experimental infection with Sf-rhabdovirus [19]. Together with the Sf-rhabdovirus sequences in the *Spodoptera litura* SL221 cell transcriptome [24], these observations suggest cell lines derived from *Spodoptera* are likely generally susceptible to Sf-rhabdovirus infection. In addition, the Sf-rhabdovirus contamination in Bm-N and BCIRL-HS-AM1 cells indicates some Bm and Hs cell lines are susceptible, as well.

Finally, considering (i) Sf caterpillars are contaminated with Sf-rhabdovirus, (ii) other lepidopteran rhabdoviruses are only distantly related [26], and (iii) several lepidopteran cell lines are susceptible to Sf-rhabdovirus infection, we conclude Sf-rhabdovirus in lepidopteran cell lines from species other than Sf most likely reflects cross-contamination in facilities where these cell lines were cultivated in proximity to Sf-rhabdovirus-contaminated Sf lines.

2.2.3. Refractory insect cell lines—Sf-rhabdovirus infectivity assays demonstrated *Trichoplusia ni* High Five™ [7], *Heliothis virescens* BCIRL-HV-AM1 [27], *Helicoverpa zea* BCIRL-HZ-AM1 and BCIRL-HZ-FB33 [28], *Anticarsia gemmatilis* BCIRL-AG-AM1 [23], and *Drosophila melanogaster* S2 and S2R+ [14, 15] are not susceptible to Sf-rhabdovirus

infection [16, 19, 87]. These results suggest Sf-rhabdovirus has a relatively narrow host range, which is most likely restricted to a few lepidopteran insect species and cell lines.

2.2.4. Mammalian cell line susceptibility—Sf-rhabdovirus infectivity assays have shown human cell lines, including A549, HEK293, A204, A549, Raji, and MRC-5 are not susceptible to Sf-rhabdovirus infection [16, 19]. Similarly, African green monkey Vero cells could not be experimentally infected with Sf-rhabdovirus [16]. We have confirmed and extended these observations and found HeLa (human), CHO (Chinese hamster), and MDBK (bovine) cells are not susceptible to Sf-rhabdovirus infection (*A. Maghodia*, personal communication). Thus, at the time of this writing, there is no evidence Sf-rhabdovirus can infect any mammalian cell lines.

2.3. Sf-RVN, an Sf-rhabdovirus-free Sf cell line

As of this writing, Sf-RVN is the only reported Sf cell line lacking any detectable Sf-rhabdovirus contamination [21]. The biotechnologically relevant properties of Sf-RVN cells, including their morphology, growth properties, response to baculovirus infection, and recombinant protein productivity are indistinguishable from those of Sf9 [21]. The finding that Sf-RVN cells did not have higher recombinant protein productivity was surprising, considering they lack the metabolic load associated with Sf-rhabdovirus contamination, which includes active viral gene transcription, translation, particle assembly, and virus shedding. On the other hand, Sf-RVN produced about 5-10 fold higher levels of infectious baculovirus progeny, as compared to Sf9 [21].

As noted above, Sf-RVN cells are susceptible to Sf-rhabdovirus infection. However, we have found Sf-RVN cells can be kept Sf-rhabdovirus-free even in facilities with a long history of Sf-rhabdovirus-contaminated Sf cells (see section 8.2 - Measures to prevent contaminating virus-free insect cell lines).

3. Trichoplusia ni cell line virus (TnCLV) / Flock house virus (FHV) (Nodaviridae, alphanodavirus)

3.1. Discovery and characteristics

Trichoplusia ni cell line virus, or TnCLV, was first described in 2007 by a group at the National Institute of Infectious Diseases in Japan [29]. Much like the discovery of Sf-rhabdovirus at Takeda, this group serendipitously discovered TnCLV when they infected High Five™ cells (a Tn cell line) with a recombinant baculovirus encoding the hepatitis E capsid protein. They found these cells produced not only the expected baculovirus and hepatitis E virus-like particles, but also alphanodavirus-like particles. Sequencing reverse transcribed RNA from these particles revealed a previously unidentified virus, which was designated TnCLV.

TnCLV is similar to Flock House Virus (FHV), a well characterized insect virus [30]. TnCLV has a bipartite genome comprising two single stranded, positive sense RNA segments (RNA1 and RNA2), similar to FHV and other alphanodavirus genomes [30]. The TnCLV and FHV RNA 1 and 2 nucleotide sequences are 89% and 78% identical,

respectively, and the amino acid sequences of the RNA1- and RNA2-encoded proteins are 83% and 90% identical, respectively [29]. This similarity is sufficient for antigenic cross-reactivity and the TnCLV capsid protein can be recognized by a polyclonal anti-FHV antiserum [29].

Unlike rhabdoviruses, alphanodaviruses are not generally considered to be mammalian pathogens [31]. Two reports indicate suckling mice can be experimentally infected by Nodamuravirus, an alphanodavirus, when transmitted by *Aedes albopictus* mosquito vectors [32, 33]. These experimental infections cause neuronal necrosis and degeneration of paravertebral and limb skeletal muscles, resulting in flaccid paralysis of the hind limbs and death within 1-2 weeks after infection [33-35]. However, except for these experimental reports, alphanodavirus infections have only been found in insects. Furthermore, vertebrates other than suckling mice (including weanling and adult mice, weanling rabbits, weanling guinea pigs, and baby chicks) did not show any signs of disease following Nodamuravirus infection [33]. As no TnCLV infectivity assays using mammalian cell substrates or animal models have been reported, the potential biohazard posed by this virus to mammals is currently unclear.

3.2. Contaminated and susceptible cell lines

Tn cell lines are used to produce one FDA-approved human biological and one biological candidate that is currently in clinical trials [2, 36, 37]. This is much less than the number of biologicals and biological candidates manufactured in Sf cells. Combined with the fact that alphanodaviruses are not generally considered to be mammalian pathogens, this perhaps explains why there has been comparatively less research on TnCLV cell line contamination and susceptibility, as compared to Sf-rhabdovirus. The experimental approach used to assess TnCLV susceptibility is essentially the same as the infectivity assay used for Sf-rhabdovirus, as described above, but a smaller number of cell lines have been rigorously assayed.

3.2.1. Contaminated insect cell lines—As noted above, TnCLV was discovered in High Five™ cells [29], and its presence in that cell line has been independently confirmed by other researchers [38, 39]. In addition, we have detected TnCLV in TN-368 [6] and Tni PRO (Expression Systems, Davis, CA), but not in Sf cells (*A. Maghodia*, pers. comm.). Thus, as of this writing, only Tn-derived insect cell lines are known to be contaminated with TnCLV.

3.2.2. Susceptible insect cell lines—At GlycoBac, we recently isolated a new TnCLV-free Tn cell line (Tn-NVN, see below). We used this cell line for infectivity assays and found Tn-NVN cells are susceptible to TnCLV infection (*A. Maghodia*, pers. comm.), which was not surprising considering other Tn cell lines have been found to be contaminated with TnCLV

Beyond Tn, one group reported cell lines derived from four other Lepidoptera, including *C. fumiferana*, *H. zea*, *S. frugiperla* and *S. littoralis*, are susceptible to experimental infection with TnCLV, while *B. mori* Bm-N4 cells are not [40]. However, there are some questions and weaknesses associated with this study. First, while it apparently focused on susceptibility to TnCLV, the authors referred to the virus tested as FHV throughout their

report. Second, no negative controls were included in the RT-PCR experiments to rule out contamination as a potential source of false positive results. Third, the inocula were not removed nor were the target cells washed after the experimental infections were initiated, which made it difficult to determine if the authors' positive RT-PCR results reflected PCR amplification of RNA from the inocula, rather than viral replication. Finally, the investigators failed to detect Sf-rhabdovirus in Sf9 or Sf21 cells, which is inconsistent with results obtained independently by several other groups (summarized above in section 2.2.1). Thus, the conclusion that insect cells derived from lepidoptera other than *T. ni* are susceptible to TnCLV should be treated with some skepticism. In this regard, our infectivity assays showed Sf cell lines, including Sf21 and Sf9, are not susceptible to TnCLV infection (*A. Maghodia*, pers. comm.).

Beyond lepidoptera, TnCLV was found to be able to infect *Drosophila* S2 cells [29], a Dipteran cell line that is also susceptible to the closely related FHV [41].

3.3. TnCLV-free Tn cell lines

At the time of this writing, six distinct TnCLV-free Tn cell lines have been produced by several independent groups. There are important differences between these lines, including their clonal status and the extent to which they have been characterized. An assessment to determine their value as potential replacements for TnCLV-contaminated Tn lines should take all of these factors into account.

3.3.1. MSU-TnT4 (TnT4)—First described in 2008, MSU-TnT4 (TnT4) is a polyclonal cell line derived from Tn embryos by a group at Michigan State University [42]. TnT4 was reported to be TnCLV-free, as determined by an RT-PCR assay specific for a portion of RNA2 with High Five™ and Sf21 cells as positive and negative controls, respectively [38, 43]. In addition, transmission electron microscopy (TEM) analysis of thin sections or density gradient fractions produced using baculovirus-infected TnT4 cells, lysates, or cell-free supernatants revealed no evidence of nodavirus particles, but found TnCLV in positive control samples produced using High Five™ cells [38]. A comparison of recombinant protein productivity using baculoviruses encoding seven different model proteins showed TnT4, High Five™, and Sf21 cells produced these proteins at similar levels [43]. Finally, the titers of recombinant baculovirus stocks produced by TnT4 cells were 10-100 fold lower than those produced by Sf21 cells [43].

3.3.2. QAU-BTI-Tn9-4s and QB-CL-B—First described in 2008, QAU-BTI-Tn9-4s, also known as QB-Tn9-4s, is a polyclonal cell line isolated from Tn embryos in a collaborative effort between the Agricultural University of Hebei and Qingdao Agricultural University [44]. QB-Tn9-4s was reported to be TnCLV-free, as determined by RT-PCR with four primer pairs designed to amplify four different fragments of TnCLV RNAs-1 and -2, with High Five™ and Sf9 cells as positive and negative controls, respectively [45]. In addition, the species origin of QB-Tn9-4s was confirmed by using randomly amplified polymorphic DNA (RAPD) assays, in comparison to Tn egg, High Five™ cell, and Sf9 cell DNA [44]. Finally, the general properties of QB-Tn9-4s have been shown to be similar to those of High Five™ cells [45].

QB-CL-B is a monoclonal derivative of QB-Tn9-4s, which was produced in a collaborative effort between Qingdao Agricultural University and the Boyce Thompson Institute and first described in 2011 [45]. As such, one would expect this Tn cell line to be TnCLV-free, like the parental line. However, to our knowledge, neither the TnCLV status nor the species origin of QB-CL-B have been confirmed. On the other hand, like QB-Tn9-4s, the general properties of QB-CL-B resemble those of High Five™ cells [45].

3.3.3. BTI-Tnao38—First described in 2010, BTI-Tnao38 is a clonal Tn cell line produced by a group at the Boyce Thompson Institute (Cornell University) [39]. This cell line was originally designated Ao38, as it was thought to be derived from the black witch moth, *Ascalapha odorata*. However, subsequent analysis showed the cell line was in fact derived from Tn High Five™ cells, not Ao. After being identified as a Tn line, Ao38 was renamed BTI-Tnao38 to reflect its correct species origin [46]. Not surprisingly, it has been found the general properties of BTI-Tnao38 resemble those of High Five™ cells. However, unlike High Five™ cells, BTI-Tnao38 contains no detectable TnCLV RNA, as determined by an RT-PCR assay specific for a portion of TnCLV RNA1 [46].

3.3.4. Tnms42—First described in 2013, Tnms42 is a subclone of High Five™ cells produced at the Boyce Thompson Institute [47, 48]. Tnms42 cells were reportedly TnCLV-free, as determined by “extensive testing” [48]. Notwithstanding this vague description, the Tnms42 cell transcriptome [47] lacks Tn-CLV sequences, indicating Tnms42 is indeed Tn-CLV-free. Unfortunately, no further information is available on this cell line.

3.3.5. Tn-NVN—Tn-NVN is a monoclonal Tn line isolated by GlycoBac, LLC, which has not yet been described in the literature (*A. Maghodia*, pers. comm.). Tn-NVN cells are TnCLV-free, as determined by nested RT-PCR assays with primer pairs targeting RNA1 and 2, with High Five™ and Sf9 cells as positive and negative controls, respectively. Tn-NVN cells were also tested and determined by nested RT-PCRs to be negative for Sf-rhabdovirus and Bm-macula-like virus (see below). Three different genes were sequenced to confirm Tn-NVN cells are derived from Tn. In addition, we found Tn-NVN cells grow well as either suspension or adherent cultures in either serum-containing or serum-free media. Like High Five™ Tn-NVN can produce at least some recombinant proteins at higher levels than Sf9 cells. Finally, we found Tn-NVN cells are susceptible to Tn-CLV infection, but this can be prevented by invoking stringent measures to control the exposure of these cells to potential sources of contamination (see section 8.2 -Measures to prevent contaminating virus-free insect cell lines).

3.4. *Helicoverpa zea* nodavirus (HzNV), another lepidopteran alphanodavirus

Although cell lines derived from Sf, Tn, and Bm are the most commonly used BICS hosts, cell lines from other lepidopteran species are used occasionally. One such cell line is Hz-AM1, which is derived from *Helicoverpa zea* (Hz) [49]. This cell line was discovered to be susceptible to a novel alphanodavirus designated Hz nodavirus (HzNV) upon infection with baculovirus stocks derived from HzNV-infected Hz larvae [50]. Although this study concluded BHK (hamster) and Sf9 cells also were susceptible to HzNV infection, this conclusion was based on RT-PCR data indicating the presence of cell-associated HzNV

RNA up to 6 days post infection. Considering the relatively short duration of this experiment and considering western blotting indicated the presence of HzNV capsid protein in infected Hz-AM1, but not BHK or Sf9 cells, the conclusion that Sf9 and BHK cells are susceptible to HzNV should be treated with some skepticism until further data become available. Finally, we note this study demonstrates the possibility of contaminating a lepidopteran insect cell line with an adventitious virus derived from live insects.

4. *Bombyx mori* macula-like virus (BmMLV, Tymoviridae; Maculavirus)

4.1. Discovery and characteristics

Bombyx mori macula-like virus (BmMLV) was serendipitously discovered in both Bm-N cells and silkworms [51]. BmMLV has a single linear 6.5 kb genomic RNA encoding 3 putative protein ORFs, and is classified in the Family tymoviridae, genus maculavirus. BmMLV can be inactivated by heat treatment (1h at 75°C), irradiation (10 kGy), and UV light (140 mJ/cm² at 254 nm) [52].

4.2. Contaminated and susceptible cell lines

There has been comparatively little research on insect cell line contamination with and susceptibility to BmMLV. This is probably due to the fact Bm cells are not used as commonly as Tn or Sf cells for recombinant protein expression, and only one veterinary biological is currently produced in Bm cells [2]. Moreover, Maculaviruses are considered to be mainly plant pathogens [53], and there is no evidence they can infect vertebrates. Thus, BmMLV is unlikely to be pathogenic for humans or other mammals.

4.2.1. Contaminated cell lines—Again, the experimental approach used to assess BmMLV susceptibility is similar to that used to assay susceptibility to Sf-rhabdovirus and TnCLV infection, but only a small number of cell lines have been rigorously assayed.

So far, the only cell line that has been clearly shown to be contaminated with BmMLV is the Bm-N cell line, in which the virus was originally discovered [51, 52, 54]. Several silkworm cell lines other than Bm-N have been screened for BmMLV by RT-PCR with primers specific for the capsid protein ORF [51] and western blotting with a polyclonal antiserum against the BmMLV capsid protein [54]. Unfortunately, these screens provided conflicting results. While lysates from several silkworm cell lines contained immunoreactive proteins of about the same size as the predicted BmMLV capsid protein, they tested negative by RT-PCR for BmMLV replicase and capsid RNAs.

4.2.2. Susceptible insect cell lines—At least one silkworm cell line, the BmMLV-free BmVF (Virus Free) cell line is clearly susceptible to BmMLV infection [54]. Another study found six lepidopteran cell lines derived from five species other than the silkworm (*C. fumiferana*, *H. zea*, *T. ni*, *S. frugiperla* and *S. littoralis*) were susceptible to BmMLV infection [40]. However, as noted above (section 3.2.2), this study had some experimental flaws, and the conclusion that cell lines from lepidopteran species other than the silkworm are susceptible to BmMLV infection should be treated with caution until independently confirmed.

4.2.3. Refractory insect cell lines—A strong body of evidence suggests Sf cells are not susceptible to BmMLV infection. Inoculating Sf9 cells with BmMLV-containing cell culture supernatant resulted in the presence of genomic and subgenomic transcripts at 3 days post infection [51]. However, no viral proteins could be detected at either 7 or 14 days post infection, and BmMLV capsid gene RNA was virtually undetectable by RT-PCR at 14 days post infection [55], indicating that BmMLV can only establish, at best, an abortive infection in Sf9 cells. Similarly, probing for the BmMLV capsid protein by western blotting in Sf9 and Sf21 cell lysates yielded negative results [54, 55]. Sf9 cells also tested negative for BmMLV by northern blotting with a BmMLV capsid DNA as the probe [51]. Thus, Sf cells appear to be refractory to BmMLV infection.

4.3. Mammalian cell line susceptibility

Nine mammalian cell lines from various species, including the human HeLa cell line, were examined for BmMLV susceptibility using a sensitive real-time PCR assay for the BmMLV capsid gene [55]. The results showed none of these mammalian cell lines could support BmMLV infection, and BmMLV capsid gene RNA was no longer detected in any of these cell lines by 14 days post infection. Thus, there is presently no evidence to suggest BmMLV can infect any mammalian cell line.

4.4. BmMLV-free Bm cell lines

Several Bm cell lines (NIAS-Bm-ao, NIAS-Bm-aff3, and SES-BoMo-15A) tested weakly positive for the BmMLV capsid protein by western blot [54], but tested negative for the capsid and replicase RNA by RT-PCR [51]. Considering these conflicting results, further testing is probably warranted before any of these cell lines should be considered BmMLV-free.

4.4.1. BmVF—BmVF (VirusFree) is a polyclonal cell line derived from *B. mori* embryos that was first described in 2012 [54]. BmVF was reported to be free of BmMLV as determined by RT-PCR assay for BmMLV capsid RNA [54]. Using the same assay, capsid RNA was readily detected in Bm-N4, but was not detected in Sf21 cells. Western blotting using a serum against the BmMLV capsid protein also showed BmVF cell lysates were negative, with a strongly positive result in control Bm-N cell lysates [54]. These same methods in combination with real-time PCR analysis of capsid RNA also showed BmVF cells are susceptible to BmMLV infection [54, 55]. Recombinant protein yields obtained from BmVF cells using Bm-NPV vectors encoding two different model proteins were found to be similar to yields obtained with Bm-N4 cells [56], suggesting BmVF cells are a safer alternative to Bm-N4 cells for biological production.

5. Arboviruses (Flaviviridae; Flavivirus)

Arboviruses are of special interest, as many are vertebrate pathogens that can infect and cause serious disease in humans. Several arboviruses can stably and persistently infect cell lines derived from mosquitoes, which are naturally permissive hosts. However, it also has been shown arboviruses including St. Louis encephalitis, Dengue and Japanese B encephalitis virus can persistently infect lepidopteran cell lines used for recombinant protein

production. As outlined below, the only examples of persistent arboviral infections in lepidopteran cell lines were reported following intentional inoculation, and there are no reports of serendipitously discovered persistent arboviral infections in lepidopteran cells.

Both wild-type and attenuated Japanese B encephalitis virus (JEV) strains can productively and persistently infect Sf9 cells [57, 58]. Sf9 cells infected with wild-type JEV had obvious cytopathic effects (CPEs) in the form of giant, multinucleated cells with granulated cytoplasm and syncytia formation, while infection with an attenuated strain did not produce these CPEs [57, 58]. Viral antigens could be detected in JEV-infected Sf9 cells by indirect immunofluorescence, however, not all cells were positive, indicating there were uninfected cell subpopulations in infected Sf9 cell cultures [57, 58]. Virus particles were detected by electron microscopy in JEV-infected Sf9 cells [58] and in cell culture supernatants concentrated by ultracentrifugation [57]. JEV appears to establish persistent infections in Sf9 cells, as cell-free supernatant and cell-associated infectious JEV virus titers were stable for at least 31 weeks of continuous culture in one study [58], and for at least 35 days in another [57].

It also has been shown that St. Louis encephalitis (SLE) virus can productively and persistently infect Sf9 cells [59, 60]. Similar to wild-type JEV-infected Sf9 cells, SLE infection induced CPEs in Sf9 cells, including syncytia, granulated, multinucleated, and giant cells. Intracellular virus particles were observed by electron microscopy in Sf9 cells infected with SLE virus and viral antigens could be detected by indirect immunofluorescence [59, 60]. Like JEV, SLE also appears to establish persistent infections in Sf9 cells, as high levels of infectious SLE virus were found in cell-free supernatant and cell lysates of SLE-infected cells continuously cultured for up to 8 weeks [59, 60].

Dengue type 1 and 2 viruses are also reportedly able to infect Sf9 cells, leading to the production of infectious virus for up to 5 weeks, but not longer [60]. Thus, despite their ability to initially replicate at low levels, Dengue type 1 and 2 viruses apparently cannot persistently infect Sf9 cells, as infectious virus eventually disappears from infected cultures [60].

Chikungunya virus has also been reported to infect Sf9 cells and produce infectious virus [61, 62]. However, as no further details were reported, it is not clear if Chikungunya virus establishes persistent infections in Sf9 cells.

6. Persistent baculovirus infection (Baculoviridae)

Baculoviruses are strictly arthropod pathogens and are not known to cause disease in any vertebrate [63, 64]. Thus, they are of relatively low concern in terms of biosafety.

Typically, infecting susceptible lepidopteran cell lines with baculovirus causes obvious CPEs and cell lysis followed by cell culture death within days. However, infection with some baculovirus mutants can produce a persistent infection [65]. Also, persistently infected lepidopteran cell cultures have been obtained using specific combinations of cell lines and baculovirus strains [66-68]. In those studies where persistently baculovirus-infected insect

cell cultures were obtained, the cultures typically went through a crisis phase characterized by high levels of cell infection and death following the initial infection.

In this context, we should also note *HZN*V-1, a Nudivirus initially classified as a baculovirus. This virus was discovered in a latently infected lepidopteran cell line [69-73]. Following a crisis phase, *HZN*V-1 could establish a persistent / latent infection in other lepidopteran cell lines, including Tn-368, a cell line used for recombinant protein production [74-76].

7. *Drosophila* cell line viruses

Drosophila cells are used for recombinant protein expression for both basic research [77] as well as therapeutic [78] applications (*Drosophila* expression system, DES). Typically, the DES employs *Drosophila* S2 cells [14], which can be persistently infected by several adventitious viruses, as outlined below.

7.1. *Drosophila* X virus (DXV, Birnaviridae; Entomobirnavirus)

Drosophila X virus was discovered in 1978 in *Drosophila* flies and *Drosophila* 68K cells [79]. DXV is classified as an Entomobirnavirus, a genus believed to infect only insects [80]. S2 cells are susceptible to DXV infection, and at least one lab strain (S2-GMR) has been shown to be persistently infected with DXV [81]. DXV does not cause CPEs in the mammalian cell lines KB, HeLa, BHK, and Vero, or in suckling mouse brains [79]. There are no reports indicating DXV can establish persistent infections in cell lines from species other than *Drosophila*.

7.2. Other *Drosophila* viruses

Several other *Drosophila* viruses can persistently infect *Drosophila* cell lines. However, it is not known if any of these viruses can infect mammalian cells or cells from any other insect species.

Three previously unknown adventitious viruses were discovered in an S2 lab strain (S2-GMR) persistently infected with DXV [81]. These included *Drosophila* totivirus (DTV, *Totiviridae*), *Drosophila* birnavirus (DBV, *Birnaviridae*) and *Drosophila* tetravirus (DTrV, *Tetraviridae*) [81]. These three novel viruses were only distantly related to viruses known at the time, and we still know nothing about these viruses except their nucleotide sequences.

A novel virus related to FHV (*Drosophila* line 1 virus, DLV) was discovered as a persistent adventitious contaminant in *Drosophila* S1 cells [82]. The authors of this study noted DLV likely arose from an inadvertent infection with Black beetle virus (BBV, *Nodaviridae*, *alphanodavirus*), which was present in their laboratory and does not cause CPE in *Drosophila* cells. Considering FHV and TnCLV can also persistently infect *Drosophila* cells [29], alphanodaviruses in general should be considered as potential adventitious viral contaminants of *Drosophila* cell lines.

Finally, several other viruses including Cricket paralysis virus (CrPV, *Dicistroviridae*), *Drosophila* A virus (DAV, unassigned (+) ssRNA), *Drosophila* C virus (DCV, *Dicistroviridae*), Invertebrate iridescent virus 6 (IIV-6, *Iridoviridae*), Sindbis virus (SINV,

Togaviridae), and Vesicular stomatitis virus (VSV, *Rhabdoviridae*) have been found to be able to infect S2 and other *Drosophila* cell lines [83].

In summary, considering *Drosophila* cell lines can be persistently infected with a wide variety of viruses, and considering they have been shown to be susceptible to mixed persistent infections with up to 4 viruses [81], *Drosophila* cells should be extensively assayed for adventitious viral contamination before being used for either biologicals production or research.

8. Conclusions

8.1. Viruses are associated with most, if not all natural insects populations

Recent studies indicate previously unknown viruses are associated with most, if not all natural insect populations [84-86]. Discovery of these diverse and ubiquitous viruses has only recently become possible by the emergence of massively parallel sequencing technologies. These viruses are well adapted, as they do not appear to cause pathology, infected populations are thriving, and they are often transmitted vertically [26]. Thus, it should be expected, *a priori*, that cell lines newly isolated from primary insect tissues are highly likely to be contaminated with one or more, possibly previously unidentified viruses. Thus, newly isolated insect cell lines should be thoroughly screened for potentially contaminating viruses, preferably using massively parallel sequencing technologies.

8.2. Measures to prevent contaminating virus-free insect cell lines

In our experience, adventitious virus-free insect cell lines can be maintained as such indefinitely if stringent measures are implemented to avoid contamination. We implemented a series of measures specifically designed to prevent our virus-free cell lines from becoming infected with adventitious viruses. In summary, we use laboratory facilities and equipment including biological safety cabinets, cell culture incubators, and pipette aids dedicated exclusively to adventitious virus-free cell culture. Furthermore, we use dedicated cell culture reagents, such as growth medium, and use disposable personal protective equipment (PPE), culture vessels, and plastic ware. We routinely and rigorously test adventitious virus-free cell cultures for the presence of viral genetic material to validate their status. Finally, we use adventitious virus-free baculovirus stocks to prevent contamination upon baculovirus infection. We have found these measures can be effective in preventing Tn-NVN and Sf-RVN cells from becoming contaminated with TnCLV or Sf-rhabdovirus, respectively, and recommend their implementation when adventitious virus-free cell lines are used.

8.3. Summary

As demonstrated by the many examples above, insect cell lines are often contaminated with adventitious viruses. In fact, one or more adventitious viruses have been found to be associated with all cell lines routinely used for recombinant protein expression. Considering this current state of the art, insect cell lines should only be considered free of adventitious viruses following rigorous experimentation including appropriate positive and negative controls over a substantial period of time.

The development of virus-free insect cell lines has resulted in new tools that allow researchers and biological manufacturers to avoid adventitious viral contamination. Ongoing work in this area is expected to further enhance the safety of biologicals produced in insect cell lines, and should also build confidence in research results obtained using insect cell lines.

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Abbreviations

BICS	Baculovirus insect cell system
Bm	<i>Bombyx mori</i> (silkworm)
CPE	Cytopathic effects
Dm	<i>Drosophila melanogaster</i> (fruit fly)
DES	<i>Drosophila</i> expression system
EST	Expressed sequence tag
Sf	<i>Spodoptera frugiperda</i> (fall armyworm)
TEM	Transmission electron microscopy
Tn	<i>Trichoplusia ni</i> (cabbage looper)
H_z	<i>Helicoverpa zea</i> (corn earworm)