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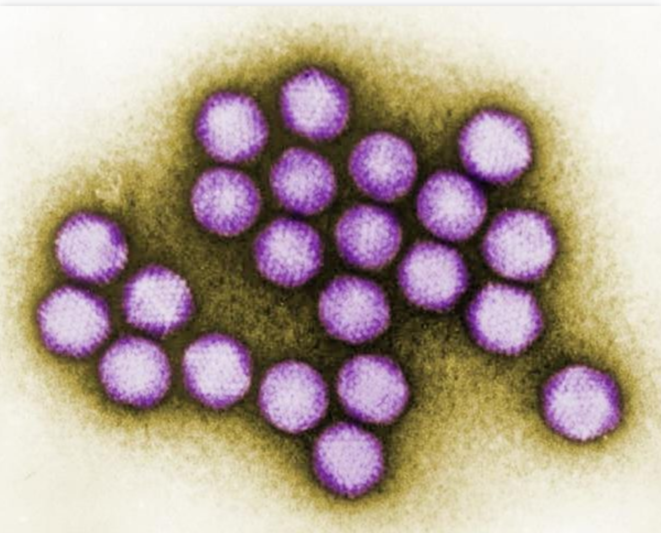
Adventitious Viral Contamination of Biopharmaceuticals: *Who is at Risk?*

By RAYMOND NIMS

Over the course of 25 years, the potential therapeutic value of biopharmaceuticals has been realized. One component of the overall safety paradigm for these therapeutic agents is the evolution and execution of viral testing, and it has been an exciting time for those of us involved in it. Participating in several of the viral contamination events that have occurred during this quarter century has proven to be particularly enlightening. Most involved hamster (especially Chinese hamster) cell substrates. This has been somewhat surprising given that biopharmaceuticals are produced in cells derived from a number of different animal species. More recently, however, viral contaminations involving human and monkey cell substrates have also been reported.

While considering risk to biopharmaceutical manufacturing processes, it is not unusual to hold the belief that a mammalian production cell is riskier, although this is not appropriate. Even processes involving bacterial fermentation are at risk of viral contamination by the bacteriophage. These agents are not believed to represent a patient safety risk but they can certainly wreak havoc with manufacturing processes utilizing bacterial production cell substrates.

Any discussion of viral risk to biopharmaceutical manufacturing should therefore include both cell culture and bacterial fermentation processes, and their associated viral contaminants.



Colorized transmission electron micrograph of adenovirus. (Courtesy of: CDC / Dr. G. William Gary, Jr., http://phil.cdc.gov/phil/home.asp.)

Introduction

The overall safety paradigm for biopharmaceuticals is more complex than that of small molecule therapeutic agents because special concern must be given to adventitious agent impurities (contaminants).[1-2] Infectious agents (*e.g.*, viruses, bacteriophage, mollicutes, bacteria, mycobacteria, fungi, mold, and transmissible spongiform encephalopathy) are introduced into the manufacturing protocol from process additives (especially animal-derived raw materials), operators, or the environment. Infectious contaminants that are endogenous in or inherent to the manufacturing process, such as endogenous retroviruses, prophage, or other latent infectious agents that are present in the production cell substrate itself are excluded from this classification. Such non-adventitious agents would include replication-competent recombinants in replication-incompetent viral gene therapy vectors, production viruses in vaccines, or oncolytic viruses.

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In this article, the following topics relative to adventitious virus contamination of biopharmaceutical manufacturing processes will be discussed:

What are some examples of adventitious viral contaminants?

How often does this type of contamination occur?

Where do viral contaminants come from and how are they introduced into the process?

What are the manifestations of the viral contaminations in the manufacturing process?

What manufacturing processes are at risk?

How can manufacturers mitigate the risk of viral contamination?

How should manufacturers respond to a viral contamination?

Examples of Adventitious Viral Contaminants of Biopharmaceutical Manufacturing Processes

Before discussing the list of known adventitious viral contaminants, it is appropriate to mention that the list includes those contaminants that we know about — because they have been publicly reported. In the past, this information has been derived primarily from three types of sources: 1) technical papers published voluntarily by companies such as Bioferon GmbH,[3] Genentech,[4] Genzyme,[5] and Boehringer Ingelheim Pharma KG[6] as a service to the biopharmaceutical industry; 2) technical papers published by contract testing organizations with summary results of testing performed over various periods of time (*e.g.*,[7-9]); and 3) press releases from companies obliged to publicly disclose contamination occurrences due to projected shortfalls in product availability to patients (*e.g.*,[10]).

More recently, biologics manufacturers have become a bit more willing to present details of viral contamination occurrences at scientific and trade conferences. This is exemplified by the revelations of an adenovirus contamination event by Eli Lilly[11] and mouse minute virus (MMV) contamination events by Amgen, Inc.[12] and Merrimack Pharmaceuticals.[13]

However, it is reasonable to assume that not all viral contamination events in production settings have been described in publicly-accessible documents or forums. An initiative entitled the "Consortium on Adventitious Agent Contamination in Biomanufacturing (CAACB)" is presently being launched by the Massachusetts Institute of Technology's Center for Biomedical Innovation with the expressed goal of uncovering this type of information in a manner that will protect the confidentiality of the information providers.[14]

The known viral contamination occurrences impacting biopharmaceutical manufacturing processes are summarized in Table 1. These are listed in the chronological order of when the contaminations were disclosed to the public. Not included are events involving cell lines outside of the biopharm production realm. Also excluded are cases in which viral genomic sequences have been discovered in products without demonstrating that these sequences represent infectious virus.

It is interesting that so many of the contamination occurrences have involved CHO cell substrates. This is likely a reflection of the frequency with which this particular cell line has been used, as well as the nature of the production processes involved (many of which have used relatively large volumes of animal serum, a known risk factor for viral contamination). In fact, the CHO cell has been evaluated by several investigators for susceptibility to viral infection and the list of viruses known to infect this cell line is not extensive.[15-18]

TABLE 1. Known viral contaminants of biopharmaceutical manufacturing processes.

COMPANY	VIRAL CONTAMINANT	CELL SUBSTRATE	REFERENCE(S)
Bioferon GmbH	Epizootic hemorrhagic disease virus	CHO	[3, 21]
Genentech	Mouse minute virus	CHO	[4]
Boehringer Ingelheim Pharma KG	Vesivirus 2117	CHO	[6]
Undisclosed	Reovirus type 2	CHO	[2,7]
Undisclosed	Cache valley virus	CHO	[2,7–8]
Genzyme Corp.	Vesivirus 2117	CHO	[5,10]
Eli Lilly	Human adenovirus	HEK293	[11]
GlaxoSmithKline	Porcine circovirus type 1	Vero	[18,20, 22–23,25]
Amgen, Inc.	Mouse minute virus	CHO	[12]
Merrimack Pharmaceuticals	Mouse minute virus	CHO	[13]

ACRONYM KEY:
Chinese hamster ovary (CHO); human embryonic kidney (HEK); African green monkey kidney (Vero)

What about bacterial fermentation processes? Although bacteriophage contamination is known to occur, to the author's knowledge, there are no case studies covering this type of contamination in the public realm. This is discussed further in the section to follow and in a recent paper by Los.[19]

How Often Does This Type of Contamination Occur?

Viral contamination of a mammalian cell-based biopharmaceutical production process is quite a rare event (though perhaps it is more accurate to say that we rarely hear about such events). In a recent presentation, Jim Skrine, Executive Director of Amgen's North American Operations/ Quality mentioned that the company had manufactured biologics for 20 years (in CHO cells) without experiencing a viral contamination — then MMV was discovered in one of their processes.[12] It is surprising that this parvovirus has not shown up more often because the root causes of its presence have been difficult to establish. The 2010 reports of MMV contamination by Amgen[12] and Merrimack[13] (Table 1) represent the first known occurrences for MMV since the Genentech experiences reported by Robert Garnick in 1996.[4]

Contract testing laboratories may evaluate several hundred biopharmaceutical lot release samples in a given year using assays such as the *in vitro* virus screen and the MMV infectivity assay (also *in vitro*). Summary reports from a decade of testing[2,7-9] reveal only a paucity of positive findings. However, when positive results *are* observed at a contract testing lab, it is not unusual to detect clusters (multiple occurrences) of contamination with one organism in adjacent lots produced by a client. This was the case for contamination with reovirus type 2 (REO)[7] and with Cache Valley virus (CVV).[7-8]

As mentioned above, contaminations of bacterial cell production processes due to bacteriophage are rarely if ever reported.[19] This is likely because the possibility of such a contamination imparts a degree of business risk to the manufacturer but little or no perceived patient risk. An infection with a lytic phage typically causes a failure of the production process to reach control limits for bacterial titer, and subsequently results in termination of the production lot. It is therefore very difficult to ascribe a number or frequency to this type of contamination event.

Where Do Viral Contaminants Come From, and How Are They Introduced Into the Manufacturing Process?

As mentioned previously, adventitious viral contaminants may be introduced from raw materials (process additives), operators, or the environment. In Table 2, the list of known viral contaminants is again displayed, this time with an assignment of the most likely source for the infection.

At this point, another disclaimer is warranted. While animal-derived materials (ADM) are implicated in most of the contamination events, in every case, the required prospective testing of the implicated ADM had failed to indicate contamination with the virus involved. Where retains of the implicated ADM have been subjected to retrospective testing using specific endpoint assays (such as polymerase chain reaction [PCR] amplification), again the testing failed to confirm the presence of virus or viral genomic sequences.

How is this possible? It is assumed that the ADM are produced in large lots prepared from multiple donor animals and that the contamination is non-homogenous across the ADM lots. Assignment of the ADM as the root cause for the contamination has typically been made by ruling out other possible sources and considering the specific biopharmaceutical production lots affected versus ADM lots used.

Examples of contaminations attributed to ADM include epizootic hemorrhagic disease virus (EHDV), CVV, REO, vesivirus 2117, and porcine circovirus type 1 (PCV1). The porcine circovirus is proposed to have been introduced into the Vero production cell substrate via porcine trypsin.[18,22-23] The required pre-screening protocol[24] for porcine-derived materials does not include a test for porcine circovirus and, in fact, this virus would not be detected using the endpoints of this screening assay (cytopathic effect, hemadsorption, and

TABLE 2. Proposed sources of viral contaminants.

VIRAL CONTAMINANT	PROPOSED SOURCE[1]	REFERENCE(S)
Epizootic hemorrhagic disease virus	Non-irradiated fetal bovine serum	[3, 21]
Mouse minute virus	Raw material or environment	[4, 12–13]
Vesivirus 2117	Fetal bovine serum[2]	[6]
Reovirus type 2	Non-irradiated fetal bovine serum	[2, 7]
Cache valley virus	Non-irradiated fetal bovine serum	[2, 7–8]
Human adenovirus	Operator	[11]
Porcine circovirus type 1	Porcine trypsin	[18, 22–23, 25]

[1]The source is that proposed by the reference document. In no case has the source been confirmed by detection of the contaminant in the specific operator or raw material involved.

[2]The references for the contamination at Genzyme[5, 10] do not propose a source. It is the author's (Nims) opinion that bovine serum was the most likely source for the contamination.

immunofluorescence). The viruses believed to have been introduced via infected bovine serum (EHDV, CVV, REO) are each known to infect cattle. The data for vesivirus 2117 are less compelling. The caliciviruses generally display a fairly broad host range, yet this particular entity has thus far been found to infect only CHO cells.[5] One must be concerned with recurrences of contamination with vesivirus 2117 as this is a very small, non-enveloped virus that has the potential to survive on facility surfaces. As with the parvovirus MMV described below, a vesivirus contamination may need to be responded to with entire facility decontamination, as was performed using vaporous hydrogen peroxide by Genzyme.

The source for contaminations with MMV has historically been difficult to establish with any certainty. This virus has contaminated manufacturing processes utilizing bovine serum, but this ADM has never been implicated as the source. Each description of MMV contamination[4, 12-13] has proposed an undetermined raw material as the possible source. Parvoviruses such as MMV are notorious for being able to survive on surfaces; therefore once an MMV contamination occurs in a facility, one must be concerned with the potential for recurrences that result from facility contamination itself. It is for this reason that entire facility decontamination[12] must be contemplated following MMV contamination of a manufacturing facility, particularly if the contamination is not confined to early upstream process steps.

The one case of contamination of a human cell production process with human adenovirus[11] was attributed to an infected operator. This is quite an unusual contamination event and the implication of the contamination is that an open vessel step must have been involved. Such operations are usually limited to very early upstream process steps.

Adventitious bacteriophage infections may be introduced through open vessel operations and through inadequate sterilization of both incoming gasses and process additives. For instance, using 0.22-micron pore size filters to "sterilize" process additives is not an effective means to mitigate the risk of bacteriophage contamination. Risk mitigation approaches appropriate for viruses are also applicable to bacteriophage and will be described in a following section. As in the case with viruses, a small, non-enveloped phage may be able to survive on facility surfaces or biofilms leading to eventual contamination of a subsequent production lot.

What Are the Manifestations of Viral Contamination In the Manufacturing Process?

Adventitious viral contaminants may or may not cause perturbations in the affected upstream manufacturing process (Table 3). The effects observed are dependent upon the growth characteristics of the virus and the type of production cell. For instance, the cytopathic effect caused by CVV spreads very rapidly throughout a CHO cell production culture. A CVV infection therefore is typically reflected by a loss of a monolayer in roller bottle CHO processes or by drastic decreases in oxygen and base demand in bioreactor CHO processes.[7-8] This is also true for an EHDV infection of a CHO cell process.[3] Adventitious bacteriophage contamination of a bacterial fermentation process also typically results in a widespread cell death that becomes apparent through process control parameters for cell titer not being achieved.

On the other hand, infection with vesivirus 2117 and MMV may cause more variable and subtle manifestations in manufacturing processes. These have ranged from no effect[4,13] to poor cell growth and viability,[6,10] and pH changes and cell death.[12] Contamination with REO or PCV1 typically does not lead to changes in production cell viability and growth or in product titer and characteristics. Reovirus infections are not typically discovered until the bulk harvest is submitted for viral safety testing.[7] In the case of PCV1 contamination of rotavirus vaccine, the presence of the virus was not detected until a new detection technology (deep sequencing)[25] became available and was applied to the vaccine product.

TABLE 3. Manifestations of viral contaminants on manufacturing processes.

COMPANY	VIRAL CONTAMINANT	MANIFESTATION	REFERENCE(S)
Bioferon GmbH	Epizootic hemorrhagic disease virus	pH change and widespread cell death	[3, 21]
Genentech	Mouse minute virus	No effects observed	[4]
Boehringer Ingelheim Pharma KG	Vesivirus 2117	Not discussed	[6]
Undisclosed	Reovirus type 2	No effects observed in cells or product	[2,7]
Undisclosed	Cache valley virus	Striking cell death	[2,7–8]
Genzyme Corp	Vesivirus 2117	Poor cell viability and growth	[5,10]
Eli Lilly	Human adenovirus	Not discussed	[11]
GlaxoSmithKline	Porcine circovirus type 1	No effects observed in cells or product	[22]
Amgen, Inc.	Mouse minute virus	pH change and cell death	[12]
Merrimack Pharmaceuticals	Mouse minute virus	No effects observed in cells or product	[13]

What Manufacturing Processes Are At Risk?

Adventitious viral contamination can occur in any manufacturing process that is based on the use of prokaryotic or eukaryotic cells. Mammalian, avian, and insect cells can be infected with adventitious viruses. Bacterial cells are susceptible to bacteriophage. Yeast cells are susceptible to yeast viruses. So the possibility of a viral contamination looms constantly over each of these biopharmaceutical manufacturing processes. It is part of the territory, and the regulatory agencies understand this.

What then, does the regulatory community expect? They expect each manufacturer to assess the risk of introducing a viral contaminant into a product and then take appropriate steps to mitigate that risk. If a manufacturer encounters an adventitious viral contamination, the appropriate response is to investigate the occurrence, sort out the root cause, and (again) to mitigate the risk of experiencing the contamination repeatedly. It is important to realize that the risk associated with viral contamination is not only a patient safety risk, but is also a business risk. Minimizing the downtime experienced following a viral contamination requires that manufacturers have a documented procedure for: 1) investigating root cause (including obtaining an identification of the contaminant); 2) detecting, segregating, and eventually disposing of affected lots; 3) containing and eradicating the infection; and 4) restarting manufacturing after the contamination investigation is closed.

How Can Manufacturers Mitigate the Risk of Viral Contamination?

The viral safety of biopharmaceutical products is accomplished through a three-part paradigm: 1) reducing or eliminating use of raw materials (especially those that are animal); 2) raw materials, in-process, and lot release viral safety testing; and 3) process engineering to limit the points of virus introduction by inactivating and/or removing viruses that might be present in upstream process additives, and to maximize the clearance of any introduced viruses in downstream purification steps. Each of the these primary components has its limitations, so they must all be combined to achieve an acceptable degree of viral safety.

As viral risk has historically been associated with the use of animal-derived raw materials,[18] reducing or eliminating the use of such materials (and for bacterial fermentation processes, reducing the use of fermentation-derived materials) represents an effective risk mitigation approach. In cases where the materials must be used, manufacturers should assess the risk associated with the material as part of an overall raw materials risk assessment program and, if necessary, subject such process additives to risk mitigation treatments. Fortunately, the list of effective viral (and bacteriophage) risk mitigation treatments has been expanding in recent years.

In the past, the primary treatments for high-risk materials have included autoclaving the materials, viral filtration, and gamma irradiation. Autoclaving a material will render the material essentially free of infectious viruses and bacteriophage. However, the use of this strategy on protein-containing process additives can denature the protein. While viral filtration using a parvovirus filter would be expected to remove all viruses, this strategy is not practical or cost-effective for all types of process additives. Gamma irradiation has historically been applied to animal sera and trypsin. It is important to realize that this strategy has limitations. In particular, very small viruses or bacteriophage are not effectively inactivated by gamma irradiation at the dose levels normally employed (25–45 kGy) so this treatment should not be viewed as a risk mitigation strategy for all viruses and phage.[18, 26]

More recently, high-temperature short-time (HTST) treatment has been characterized as a means of adventitious agent risk mitigation for upstream process additives, including cell culture media. This technology is a modification of the pasteurization treatment. Schleh *et al.*[27-28] and Weaver and Rosenthal[29] have found HTST treatment (102–115 °C for 10–30 seconds) to be an effective means of inactivating the parvovirus MMV and the bunyavirus CVV in cell culture media. Another advancement is the use of ultraviolet radiation in the C range (UV-C; λ max ~254 nm; fluency ~125 mJ/cm^2). This approach has been demonstrated to effectively inactivate the parvovirus MMV and the bunyavirus CVV in cell culture media[28-29] and inactivate a variety of enveloped and non-enveloped viruses including porcine parvovirus and simian virus 40 (SV40) in protein solutions.[30] The primary advantage of each of these technologies is that they represent an effective and robust means of inactivating the smaller non-enveloped viruses and can be operated in a flow-through manner for large volume process additives such as media and buffers.

Replacement of ADM with materials derived from plants may reduce (but not eliminate) the viral risk associated with the process additives.[31] Plants can serve as surfaces for small non-enveloped viruses derived from wildlife and small non-enveloped bacteriophage. As mentioned above, some of these viruses (especially the parvoviruses and picornaviruses) and phage may survive for long periods of time on such surfaces. If the processes used to derive the additives from plants are not stringent, contaminating viruses or phage may make their way into a manufacturing process via the plant-derived additive.

Process additives derived from bacterial fermentation do not represent any significant risk for introducing viruses into animal cell production processes. Bacteria are not capable of supporting the proliferation of viruses (other than bacteriophage). Additives derived from bacterial fermentation could, theoretically, introduce bacteriophage into a biologics manufacturing process employing bacterial production cells. It is not typical, however, for fermentation-derived additives to be used in this type of manufacturing process.

In general, applicable risk mitigation strategies for upstream process additives include viral filtration, HTST, and UV-C treatment. Testing of ADM used as upstream process additives is required (9CFR113),[24] yet the testing that is typically performed by both raw material suppliers and the end user has limited value as a viral risk mitigation strategy.

As mentioned earlier, the reasoning is that the high-risk animal sera and trypsin are typically produced in large lots utilizing multiple animals, and the donor materials are not always homogeneously distributed throughout the entire lot. For 9CFR113 testing, one or two bottles out of an entire lot may be sampled. A negative (passing) result for the sample tested is not an absolute representation of the entire lot's lack of adventitious contaminants.

How Should Manufacturers Respond To a Viral Contamination Event?

The risk of viral contamination for a manufacturing process can be reduced but not entirely eliminated. When a manufacturing process has been contaminated with a virus or bacteriophage, it is critical to contain the infection so that entire upstream/downstream processes and associated equipment do not become contaminated. Key to effective containment is a facility designed to segregate these operations and the use of in-process testing results as a means of discovering a viral or phage contamination at the earliest possible step. Two in-process tests that have traditionally provided an early indication of a viral contamination include: 1) cell (or bacterial) growth and viability; and 2) demand for oxygen and pH adjustment additives.

Following an adventitious viral contaminant event, regulatory agencies may expect the manufacturer to add an in-process test for the specific contaminant involved. For example, Genentech, Amgen, and Merrimack each implemented in-process MMV PCR assays to provide an early indicator of any subsequent contamination with that virus in their upstream processes.[4,12-13]

Additional mandatory responses following a viral contamination might include: 1) the assessment of lot release testing to enable detection of the specific virus involved in the contamination; 2) the assessment of cleaning procedures for effectiveness in inactivating the specific virus; and finally, 3) a reassessment of viral purification processes for effectiveness in removing and/or inactivating the particular virus.

For bacteriophage contamination events, it may be necessary to obtain some information regarding the specific phage involved. Key considerations are the particle size and envelope status as these characteristics may indicate the business risk associated with the infection. As with other viruses, bacteriophage that are small and non-enveloped may contaminate the facility and threaten future production runs. A bacteriophage contamination occurrence in a bacterial fermentation process may require manufacturers to reassess their cleaning procedures and consider the necessity for facility decontamination. The same methodology used in handling a viral infection in an animal cell production process could be implemented in a biologics manufacturing process employing bacterial production cells.

Summary

The manufacture of biopharmaceuticals using prokaryotic and eukaryotic production cell lines has an associated risk of viral or bacteriophage contamination. This risk is understood by the regulatory agencies and is addressed in the various guidance documents applicable to this industry. The viral safety paradigm for biopharmaceutical production, including raw material screening, viral detection testing, and process engineering and viral purification process characterization and validation, has been put in place to protect patients from exposure to an infectious contaminant via administration of a biological product. Business risks to biopharmaceutical manufacturers are also mitigated by various aspects of this safety paradigm. Patient/business risks are therefore addressed jointly through assessing and mitigating the risk for viral contamination of a manufacturing process environment.

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