A Publication of the
WilBio Institute for BioProcess Technology

Spring 2008
ISSN 1538-8786

BioProcessing JOURNAL

Trends and Developments in BioProcess Technology

Vol. 7/No. 1

www.bioprocessingjournal.com

Virus Safety Testing for the Next Generation of Biologics

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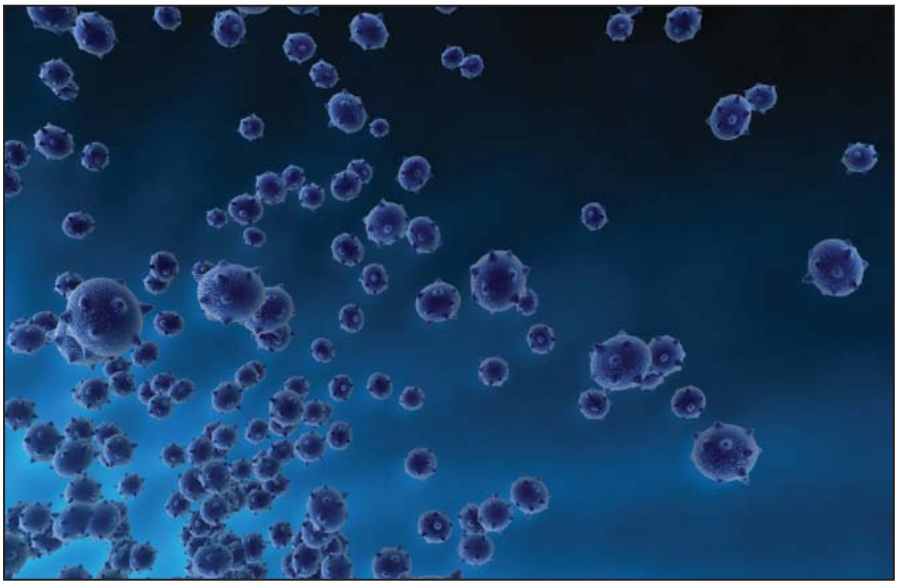

Today, many if not all of the major pharmaceutical companies have specific budgets for large research and development pipelines for new biologics including vaccines, monoclonal antibodies, recombinant proteins and even gene therapy projects. In addition, small innovative companies are continually presenting us with novel treatment opportunities which hold great promise for the future. The reason for such huge optimism in the field of biologics is that there are now a number of "blockbuster" drugs for which the safety profile has been very successfully managed.[1] This success has been achieved following a considerable amount of time and financial investment by both the innovators and the regulators.

There are a number of hurdles to overcome for new biologics. A very important one involves the potential for viruses to contaminate the production process. The safety of a new drug is always of paramount importance, but with the experience gained over the past 30 years, perhaps we are at a juncture where the established virus safety testing protocols for new products should be reviewed.

The reason behind the need for virus safety testing of biologics can be traced back to the introduction of the first mass-vaccine products manufactured during the second half of the 20th century. These products were used to prevent viral diseases which were a major cause of pathology at the time. Such vaccines were the first medicines to be produced on a large scale using cells as the basis of the production system. The best example is polio vaccine which is probably one of the greatest success stories of our time. Mass-vaccinations have succeeded in controlling and virtually eliminating this disease.

The cells used in polio vaccine production were originally prepared from animal organs. Back then, many of the donors were sourced from uncontrolled populations with very little screening for health status. There is published evidence that adventitious viruses present in the animal cells were introduced into the polio vaccine.[2]

There have been a number of vaccines and products manufactured from human blood or blood derivatives which have resulted in the transmission of adventitious agents (particularly viruses) to the recipient. All of these incidents led to the creation of testing guidelines for vaccines which were implemented in Europe and the United States to ensure patient safety. With improvements in aseptic techniques and air handling and a general lifting in cGMP standards for the production of drugs came huge improvements in the safety of these products.

With the advent of recombinant DNA technology in the 1980s, proteins could be manufactured to order. This offered the opportunity to treat disease using a completely new array of techniques. Cell culture methods proved to be extremely useful as cells could be transformed with the appropriate gene of interest and then frozen in banks to be recovered for production when required. The use of cells is similar to that of the vaccine industry, however there were considerable differences with

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the safety profile of these cells when compared with primary cells sourced directly from animals.

It should also be noted that the regulators who were providing guidance to the early biologics manufacturers were presented with a product that had not been manufactured in the aseptic stainless steel facilities now used to manufacture recombinant proteins or monoclonals. Some of the first monoclonals were produced using mouse ascites fluid, a technique reliant on animals and very prone to contamination. Virus safety testing standards for these new products were based on guidance designed for the only other cell-derived products being manufactured—vaccines.[3,4] Much of the regulatory guidance for biologics production involves virus detection techniques that have been around for some time, as far back as the 1960s or earlier.

The safety testing for biologics uses a three-staged approach to assure safety: 1) testing the starting materials such as the cells and media used in the production; 2) testing of "in-process" samples once the cell culture period has ended; and 3) testing the virus reduction capacities of the downstream processing. The first two parts are similar to the safety testing strategies laid out in the vaccine guidelines of the European Pharmacopeia and the United States Code of Federal Regulations. The third step is clearly not useful in the manufacture of a virus vaccine.

The virus testing strategies described in almost all regulations for biologics splits viruses neatly into three areas: 1) the testing for adventitious viruses; 2) the testing for retroviruses; 3) and the testing for species-specific viruses. Testing for adventitious viruses covers those which are likely to be introduced in either the starting materials or as a breakdown of the aseptic protocols used during the manufacturing process. Retrovirus testing is normally concerned with the endogenous retroviruses which infect the production cell line. Testing for species-specific viruses is again concerned with viruses inadvertently introduced from the production cell line.

Despite these three neat areas, viruses themselves do not fall easily into one specific category—some viruses can be considered both adventitious and species-specific or retrovirus. Guidelines specify the types of testing necessary to fulfil the safety requirements. They normally include two tests (*in vitro* and *in vivo*) which have the ability to detect a broad range of adventitious viruses. The *in vitro* test is a cell-based assay which detects viruses by inoculating a sample onto particular "detector cells" that are susceptible to adventitious viruses. The types of cells (such as Vero or MRC-5) considered suitable for use are specified in the guidelines. Samples are observed over a specified period of time for the presence of cytopathic events which can signal the presence of viruses. The *in vivo* assay is similar to the *in vitro* method but rather, samples are inoculated into laboratory animals (Guinea pigs, adult or suckling mice, and embryonated chickens eggs) and subsequently observed for pathological markers indicating virus infection.

Virologists have been using these types of assays since the turn of the 1900s and they are still popular today. The assays are generally simple to carry out but require a very high level of expertise to determine the presence or absence of virus in a test sample. One difficulty in interpretation is that many biologic drugs cause sample-related pathology and abnormal conditions in the test animals or cell cultures.

A major issue with assessing the potential risk of contamination posed by most viruses is that there has been so little work carried out on the vast majority of viruses and their inherent ability to infect cells used in production such as Chinese hamster ovary (CHO). Most risk strategies are based on what is known about a relatively few model viruses. An example of where this model theory fails is that of *Bovine polyomavirus* (BPyV), once thought to be a high risk for contamination of production cell lines due its ubiquitous nature and the use of fetal calf serum (FCS) to supplement growth media for cell culture.[5] BPyV viruses are a well-known risk for the contamination of laboratory cell cultures. Hence, it was thought that should BPyV be present (contaminating cell cultures in the typical polyoma pattern of infection), the *in vitro* assay would identify it. However, *in vitro* culture of the virus described an unusual pattern of growth which included a long period of latency for the virus without identifiable replication. This suggested that infection of cells would not be identified using the tried-and-tested techniques and additionally—maybe there were other viruses that would not be identified using the familiar techniques.

Retrovirus testing can become a complicated regimen under the current guidelines and requires a number of techniques to be applied to satisfy the regulations. Retroviruses are perhaps one of the most interesting virus families yet described. They are ubiquitous agents present in all animal species investigated thus far. Their ability to integrate into the host cell genomes and remain silent does present a number of difficulties when testing for their presence. There are three strategies used to detect retroviruses: 1) molecular techniques to detect the present of the reverse transcriptase enzyme, an enzyme common to all retroviruses but absent in almost all other viruses; 2) electron microscopy to observe the virus particles; and 3) cell culture techniques used to detect the virus by infection of cells and subsequent cell pathology.

Retroviruses, although ever-present in all of the common production cell lines (CHO and NS0), do have a considerable disadvantage with respect to their ability to be transmitted. They are very easily inactivated when outside of the host cell and in adverse conditions and typically have very specific requirements related to the cell receptors to which these viruses will bind.

The final viruses to consider are termed "species-specific viruses" which may be introduced dependant on the type of the production cells or any animal-derived reagent used. Testing for these requires an array of techniques which are targeted at specific viruses or

virus families. These techniques can be prescriptive such as the mouse antibody production (MAP) assay to test viruses which may be present in mouse cells or be generic such as the polymerase chain reaction (PCR) assay used to detect the presence of viruses in a human production cell line.

Using the CHO cell as an example, a testing package for regulatory submission in Europe or USA is described in Table 1. This example includes an assay to determine any specific bovine viruses (assuming the cells have been cultured in media containing FCS some time in their history), and an assay to determine any specific porcine viruses (assuming the cells have been passaged using porcine trypsin). A package of work such as this would normally take 6-8 weeks to complete for the master cell bank (MCB) and around 4 weeks for an "in-process" test.

TABLE 1. An example of a CHO cell production testing package for regulatory submission.

Assay	MCB	In-Process Testing
In vitro assay (MRC-5, Vero, hamster cell)	✔	✔
In vivo assay	✔	
Electron microscopy – cells	✔	
Electron microscopy – virus particle count		✔
Retrovirus infectivity assay	✔	✔
In vitro assay to detect porcine viruses	✔	
In vitro assay to detect bovine viruses	✔	
Detection of MMV by PCR		✔
MAP/hamster antibody production (HAP) assay	✔	

When considering a risk-based strategy for a product manufactured on CHO cells, the MCB presents the highest risk of contaminating viruses as typically these are cells which have been in a less controlled environment than that of cGMP manufacturing. Cells may have been cultured near other cell lines, some of which may have been expressing endogenous or other viruses. The reagents used to culture the cells may not have been quarantined, tested and released as in a cGMP environment and therefore may have contained viruses.

Evidence has shown that the highest risks of virus contamination are related to: 1) the use of animal products in the process; and 2) as a result of a breakdown in cGMP, allowing contamination by operators or other external sources. Viruses have been introduced following the use of bovine products during production which can be considered an extremely high-risk practice. Inadequate personnel training or lapses in cGMP compliance have also resulted in compromised product integrity. Publications have described that, in addition to the contaminating viruses from FCS, other kinds of virus infection in production processes have been noted[6]—one in particular is *Minute mouse virus* (MMV). This virus has been shown to be transmitted to CHO production from wild mice. This may be considered to be a breakdown of cGMP as appropriate precautions were not undertaken to ensure the integrity of materials used in production.

Therefore, the production process should have a viral risk assessment to ascertain when and where these hazards might enter the process. To be a risk to the product, viruses must be able to survive and replicate in the environment provided, otherwise they will be inactivated or diluted out during passage. A prerequisite for virus replication is that it must be able to attach to and enter the host cell—in the preceding example, CHO cells.

To be "successful" in sustaining an infection, a contaminating virus will use one of three strategies: 1) continuously replicate in the production cells; 2) integrate into the DNA of the host cell such as accomplished by the retroviruses and some Parvoviruses; and 3) have the ability to remain latent (such as members of the *Herpes* virus family) where it can remain in cell culture without causing significant cell pathology but retain sufficient viral functions to maintain infection.

Detecting a replicating virus can be relatively easy. As in the case of most viruses, an infection of cells results in a cytopathic effect that be identified by the *in vitro* assay. When attempting to detect viruses which are slow-growing or do not exhibit cytopathic effect on infection, this can prove difficult. Retroviruses fall into this category but have their own suite of tests to detect their presence or absence. The difficulty comes when trying to detect the presence of an integrated virus.

By their nature, retroviruses can remain integrated without expression of viral proteins. Detecting such viruses requires molecular techniques designed to target the genome. Latent viruses require specific detection assays and some must be induced out of the latent stage for detection to be successful. The *in vivo* tests and other assays are not capable of detecting the presence of these inapparent viruses. In many cases, the use of cell- or animal-based assays do not provide the solution.

There are technologies such as molecular-based techniques which can be applied to detect the presence of viruses without the observation approaches of *in vitro* and *in vivo* assays. A simple process which has become very commonplace is the polymerase chain reaction (PCR) technique. All viruses for which there is sequence known can potentially be identified using PCR. One limitation to PCR is

that only a single virus type (or rarely, a virus family) can be detected using this single assay. This would make attempting to detect all possible viruses which can infect CHO cells for example, an immensely time-consuming and costly task.

While this does pose a challenge for molecular-based techniques, now there are microarray techniques which can be targeted to thousands of genetic sequences to detect a wider spectrum of viruses. The overall sensitivity of these detection methods remains to be defined. Techniques are already being published which describe the viral detection methodology based on a combination of viral genomics and long oligonucleotide DNA microarray technology. Highly-conserved sequences within a viral family have been used on the microarray, and as such, unidentified or newly-evolved virus family members could be detected. Coupling this detection to the amplification of the target region by PCR would increase the sensitivity of the assay to close to 100 copies of virus or less in a test sample.

Another especially interesting technique involves adapting the cell used in the *in vitro* assay to detect the contaminating viruses. Cells can be manipulated to signal infection of virus rather than relying on a more obvious cytopathic effect. Following virus infection, cells will initiate a number of intra and extracellular signals—one of the most widely studied being the interferon cascade. If a marker gene such as luciferase were coupled to the switching on of such genes then these cells would signal the presence of virus infection very early on, and even latent viruses could be identified in this way. The choice of cells would also be important because depending on the cell receptors available on the production cells, this will dictate the viruses which are able to enter and replicate in these cells. The production cells could be modified using the reporter gene and then used as a detector cell line to indicate virus infection or alternatively, the traditional cell types used to detect viruses such as Vero or MRC-5 cells could be used.

In Conclusion

Viruses are a known risk factor for biologics produced on cell lines. The number of agents being identified is ever-increasing—along with the associated risks which require assessment. The techniques we have used since the inception of vaccines in the 1950s have been successful in ensuring the safety of the majority of products. In this, there is no doubt. However, as we move to the future and treat many more patients with an increasing array of novel drugs, the chances of a mistake or breakdown in-processes become ever more likely. With respect to the viral risk of these products, the detection techniques we have been using provide a fair degree of reassurance. The biologics industry is now aware that there are additional techniques which can be applied to provide us with increased reliability. *Should we begin to remove the requirement for some of the traditional techniques which may have been important at one stage but have now outlived their usefulness?* Through risk-based analysis and thorough validation of the new techniques, older protocols could be replaced. This will improve the industry's profile with the wider society and maintain the highest levels of safety and integrity in our products.

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