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**Introduction**

Screening of biological pharmaceuticals for adventitious agents, particularly viruses, is a base concern in determining the safety of the products. Of the four methodologies used for detection of such agents; transmission electron microscopy (TEM), molecular based tests, *in vitro* and *in vivo* testing, it is the *in vivo* test which has recently come under most scrutiny. There are two reasons for this; firstly, there is a will, indeed a requirement, to implement the 3Rs of replacement, reduction and refinement of animal testing in all aspects of testing medicines,\(^1,2\) secondly, particularly in the case of virus detection, are the *in vivo* methods effective? \(^3\) Rebecca Sheets gives a lucid account of industry experience in regard to the utility of *in vivo* testing.\(^4\)

We would agree with Dr Sheets that much of the *in vivo* testing used for screening biologics for adventitious viruses is simply a tick box exercise that is better done using alternative strategies. The animals used are not sensitive detection systems for the likely agents we would expect to find in biologicals. In many cases the drug itself causes adverse reactions in the animals making root cause analyses of these adverse effects versus infection, complex. We focus here on the Pharmacopoeia *in vivo* test method in mice, guinea pigs and embryonated hen’s eggs.

**Arguments against change:**

Broadly, the *in vivo* test using mice, guinea pigs and hen’s eggs is based on proven methods for isolation and amplification of a range of viruses of interest and is potentially a “catch-all” able to detect viruses that may not be detectable using other methods. One of the strongest arguments for the retention of the test, in mice at least, is for the detection of a sub-group of the enteroviruses; Group A Coxsackie viruses. These viruses when isolated from clinical samples, are seldom able to grow in cell culture unless a panel of cells are used and isolation is best achieved in suckling mice.\(^5\) The “gold standard” for diagnosis and identification of human enteroviruses is nevertheless, based on cell culture with a panel of susceptible cells and molecular methods.\(^6\) When we are considering adventitious viruses likely to be present at detectable levels in biological products it should not be forgotten that the virus must be able to grow efficiently in the cell substrate, if it cannot it will be diluted from the culture and eventually disappear. Based on our industry experience and that of others\(^3,4\) *in vivo* tests have not been proven to be effective in the detection of adventitious agents in products indicating that either the risk is very low or the test simply not sensitive. Furthermore, many of the adventitious viruses that have been uncovered in biologics would not have been detected using the *in vivo* tests.\(^3\)

**Arguments for change:**

We have mentioned in the anecdotal evidence above that isolation of an adventitious virus solely by *in vivo* test alone has never been reported. Is that a reflection of the sensitivity of the test itself? Is it the result of the utilization of well characterized cell
banks for production of biological molecules thereby reducing risk? The reduction in the animal sourced materials such as early isolate primary cells or use of bovine serum in cell culture? The fact that in order to be an issue or even detect a virus, or other adventitious agent, it must firstly be able to replicate efficiently during the production cycle on the cell substrate and not be attenuated by such replication cycles? The host tropism of the virus for animals has not been used in a standard in vivo test? It is likely that any of the factors listed above contribute to the apparent ineffectiveness of the test.

The volumes tested in a standard in vivo test are, as alluded to by Rebecca Sheets, nearly always based on the minimum requirement outlined in various guidelines. In our opinion this area is “the elephant in the room” that is rarely considered; let’s consider the testing in adult mice. In the standard in vivo test, a typical test would assay 11 mL of product: 20 mice each inoculated with 0.5 mL via the intraperitoneal route and with 0.05 mL via the intracerebral route. Using a simplified equation for 95% confidence of detection:

\[ V_t = -\frac{1}{x \text{ virus/mL}} \ln(1-0.95) \]  
(Where \( V_t \) is volume tested and \( x \) is virus number)

The test might be expected to only detect a virus with 95% confidence if there were 25 infectious particles per mL even if we assume that the efficiency of infection is 100% and 1 infectious virus would be detected. If for arguments sake we apply the standard for replication competent retroviruses of detecting 1 particle in 100 mL we would need to test approximately 300 mL and that would require inoculation of approximately 545 mice. The number for suckling mice rises to over 2727 animals. While these numbers may seem extreme and testing these volumes would be challenging even using in vitro systems and very challenging for PCR they do focus us on what is an acceptable detection level and where to target the testing.

The results of a recent study designed by a panel of experts to compare in vitro and in vivo tests for detection of potential viral contaminants of biological products in a more systematic way, certainly is timely and clarified some of the issues. The design of the comparison used a range of sixteen viruses at various dilutions looking at the limit of detection on a range of up to four cell lines typically used for in vitro screening for adventitious viruses. In brief summarizing some of the results reported; all of the viruses were detected in vitro, not surprisingly, since the stocks were produced on cells. Many of the viruses were detected at levels below that of the stock quantified on the production cells, thought to be in part, a reflection of the extended length of the in vitro test compared to titration. The cell types did display different limits of detection for some viruses. Out of eleven viruses tested in the in vivo test only six were detected and out of these six, four were only detected at inoculums of 10 fold or more virus than in vitro. Two viruses, influenza and vesicular stomatitis virus, were detected in eggs at lower amounts in vivo than in vitro. Interestingly, passage of materials from previously inoculated eggs and suckling mice did not increase the frequency of detection indicating that there was no amplification of low levels of virus that may be present in the first passage material. The authors made no claims for the ability to detect adventitious agents in the presence of the biological products and as in all cases qualification of test materials in the screening is important.
Solution/Proposals

The data presented by the panel of experts, although by no means exhaustive, supports our speculation that part of the reason for the apparent ineffectiveness of standard in vivo testing is the result of insensitivity of the animals models used.

We believe that continuing to support the data generated by a standard in vivo test as an adjunct to a considered, risk based, detector cell and molecular strategy is flawed. Flawed, not only with regards to the 3Rs but more importantly because utilizing the in vivo test, even as part of an expanded testing panel, is not the best strategy to screen biological materials for adventitious agents derived from more controlled processes than previously. Risk management through risk assessment of the product and the production system including all reagents allows a more measured design of testing strategies. In many cases an extended range of cells with suitable endpoints, including TEM would be prescribed for in vitro testing reflecting the origin and history of the product, the cell substrate, biologically derived products used in its production and potential agents introduced from the manufacturing environment. This, in conjunction with a selected panel of molecular based tests such as PCR, targeting high risk agents that may not be easily detected in cell based testing would give a high level of comfort that the product is free from a majority of adventitious agents. An important part of the risk management is that these systems are amenable to qualification and are scalable to allow testing of statistically significant volumes of material.

Conclusion

The current routine use of standard in vivo test is not effective and not the best use of resources for testing for adventitious agents in risk managed products. If we applied 3Rs it would not be used for routine testing. At worst the passage of material in both embryonated eggs and sucking mice is redundant.

We would recommend the use of a panel of in vitro cell and molecular testing designed after careful risk assessment of all components in the production system.
References

1. European Medicines Agency (2011). Statement of the EMA position on the application of the 3Rs (replacement, reduction and refinement) in the regulatory testing of human and veterinary medicinal products.

2. US Public Law 89-544 1966 (The Animal Welfare Act or Laboratory Animal Welfare Act)


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