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Effectiveness of mouse minute virus inactivation by high temperature short time treatment technology: A statistical assessment

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ABSTRACT

Viral contamination of mammalian cell cultures in GMP manufacturing facility represents a serious safety threat to biopharmaceutical industry. Such adverse events usually require facility shutdown for cleaning/decontamination, and thus result in significant loss of production and/or delay of product development. High temperature short time (HTST) treatment of culture media has been considered as an effective method to protect GMP facilities from viral contaminations. Log reduction factor (LRF) has been commonly used to measure the effectiveness of HTST treatment for viral inactivation. However, in order to prevent viral contaminations, HTST treatment must inactivate all infectious viruses (100%) in the medium batch since a single virus is sufficient to cause contamination. Therefore, LRF may not be the most appropriate indicator for measuring the effectiveness of HTST in preventing viral contaminations. We report here the use of the probability to achieve complete (100%) virus inactivation to assess the effectiveness of HTST treatment. By using mouse minute virus (MMV) as a model virus, we have demonstrated that the effectiveness of HTST treatment highly depends upon the level of viral contaminants in addition to treatment temperature and duration. We believe that the statistical method described in this report can provide more accurate information about the power and potential limitation of technologies such as HTST in our shared quest to mitigate the risk of viral contamination in manufacturing facilities.

1. Introduction

Viral contaminations of production scale mammalian cell cultures are rare events as GMP manufacturing requires high standard controls of raw materials, personnel, and manufacturing processes. Unfortunately, recent cases reported by Genzyme, Amgen, and Merrimack remind us that such rare events do occur [1–3]. Complete shutdown of the facilities is often needed for root cause investigation and decontamination, resulting in significant adverse impact on both business and patients. However, root cause investigations usually fail to definitely determine the source and/or route of the viral contaminants for several reasons. Firstly, culture samples from seed train stage expansion are not normally retained; making it impossible to track when/where virus contaminant is introduced into the process. Secondly, ubiquitous nature of viruses and complexity of the production scale cell cultures imply that viral contaminants can originate from environment, personnel, medium components, or air supply, making it impractical to investigate all potential "suspected sources". Finally, direct testing is often hindered by assay sensitivity as well as non-homogenous/discrete distributions of viral contaminant in a given lot of raw material.

It is generally agreed that raw materials, those of animal sourced in particular, represent the most likely source of viral contaminants [4]. High temperature and short time (HTST) treatment of culture media has been considered as an effective barrier to prevent introduction of viral contaminants into production bioreactors. Laboratory scale studies are typically carried out to assess the effectiveness of HTST in inactivating viral contaminants and potential impact on culture media prior to implementation. Log reduction factor (LRF) has been broadly used to measure the effectiveness of chemical or physical methods for viral inactivation including HTST treatment [5–7]. Since a single infectious virus is potentially sufficient to cause a wide spread contamination once introduced into a cell culture process, LRF may not be able to accurately assess the effectiveness of HTST treatment. For example, HTST treatment reduces viral contaminants in a batch of medium from a total of 6 $\log_{10}$ (1 million) to less than 1.5 $\log_{10}$ (<31 infectious virus particles remaining). However, the surviving virus particles will still be able to initiate the infection and result in a full

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blown contamination. Therefore, despite achieving significant LRF ($\geq$4.5 $\log_{10}$), HTST treatment is not effective in preventing the contamination events in this particular scenario.

We described here an alternative approach to assess the effectiveness of HTST treatment in mitigating the risk of viral contamination. In comparison to the conventional high titer spiking and LRF approach, we believe this approach is more appropriate and representative for assessing the effectiveness of virus inactivation by HTST technology. This is because inoculation of "mini bioreactors" with HTST treated MMV-spiked medium allows statistical estimation of the treatment effectiveness as measured by achieving 100% inactivation of the input viral contaminants. In addition, viral contaminant level is usually low and discrete, thus low level spiking resembles more closely the real-life scenarios encountered.

2. Materials and methods

2.1. Virus strain and cell lines

Mouse minute virus prototype (MMVp) Crawford strain was obtained from ATCC (ATCC VR-1346). An SV-40 transformed human newborn kidney cell line (NB 324K cells) was obtained from Yale University. Mouse embryo fibroblast (MEF) cell line was established at Lilly Research Laboratories. Both MEF and NB 324K cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT).

2.2. MMV propagation and titration

MMV was propagated in MEF cells and subsequently purified by solvent extraction and ultracentrifugation. Briefly, MEF cells in T_{150} flasks (Corning, Lowell, MA) at approximately ~60% confluency were infected with MMV at a multiplicity of infection (MOI) ~0.12. After an hour adsorption at 37 °C, the inoculum was removed and the T_{150} flasks were fed with 25 ml/flask DMEM containing 10% FBS. The infected flasks were incubated at 37 °C incubator with 5% CO_2 until the development of apparent cytopathic effects (CPE), and then subjected to three cycles of freezing/thawing to release potentially cell-associated virus particles. The resulting cell lysates (~600 ml) were mixed with 350 ml Vertrel XF (Micro Care Corporation, New Britain, CT). After overnight stirring, the mixture was subjected to centrifugation at 2000 rpm (Beckman JS-5.2 rotor) for 10 min at 4 °C. The aqueous phase was collected and viruses were concentrated by centrifugation at 30,000 rpm (Beckman SW 32 Ti rotor) for 2 h at 4 °C. The pellets were re-suspended in 50 ml phosphate-buffered saline (PBS, pH 7.4). The resulting suspension was subjected to a brief centrifugation at 2000 rpm (Beckman JS-5.2 rotor) for 5 min at 10 °C to remove cell debris. The supernatant was then subjected to second round of centrifugation through a 5.0 mL 30% sucrose cushion at 30,000 rpm (Beckman SW 32 Ti rotor) for 2 h at 4 °C. The Pellets were re-suspended in 50 ml Tris-buffered saline (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.5% BSA, pH 7.5) and briefly sonicated. The sonicated virus suspension was centrifuged again at 2000 rpm (Beckman JS-5.2 rotor) for 10 min at 10 °C to remove remaining debris. The resulting supernatant was filtrated through a 0.22 μm filter (Millipore, Billerica, MA) and aliquoted. All virus aliquots were stored at ≤ -60 °C until use.

A $TCID_{50}$ assay was used to determine the MMV titer. Briefly, 96-well plates were seeded with 200 μl/well of 2.5 × 10^4/ml NB 324K cells and incubated at 37 °C overnight. The plates were then inoculated with a serial of 10-fold diluted testing sample (50 μl/well) and a total of 8 wells were inoculated for each dilution. The plates were examined well-by-well under a microscope for CPE after 10-day incubation at 37 °C. The $TCID_{50}$ titer was calculated using the Kärber formula.

2.3. Evaluation of HTST treatment

Fig. 1 schematically outlines the experimental design and execution of the evaluation study. Briefly, Eli Lilly's proprietary medium LM7105 was spiked with MMV and then subjected to HTST treatment with various temperatures and durations. The treated LM7105 were then used to inoculate NB 324K cells in T_{25} flasks which served as "mini bioreactors". It is worth noting that these "mini bioreactors" mimic actual commercial bioreactors only in a sense they allow MMV that have survived HTST treatment to propagate in susceptible cells, and thus manifesting a contamination event that can be readily detected. These "mini bioreactors" were kept in a 37 °C incubator with 5% CO_2 for 10 days and then examined for the presence of CPE under a conventional microscope. Cell lysates were harvested from each individual "mini bioreactor" after three cycles of freezing/thawing. The harvested lysates were then used to inoculate fresh NB 324K cells to determine whether all the initial CPE negative "mini bioreactors" were free of MMV. Detailed design and execution of the evaluation are described below.

2.3.1. Apparatus for HTST evaluation

The microchannel reactor (MCR) and the associated equipment selected from AFRICA microflow system (Syrris Ltd, Royston, UK) were used in this laboratory scale HTST study. The same system has been used in our laboratory in the past for the evaluation of virus inactivation by heat treatment [8]. The system is based on microfluidics technology that permits precise control of key process parameters in reactive streams in microchannel flow geometry. Employing standard microfabricated techniques, the channels with micrometer dimensions are etched on the surface of a flat plate and covered by a second plate. The space sandwiched between the two plates forms a continuous chamber in which specific reactions are allowed to occur. Fluid flow in the microchannel reactor is almost always laminar and mixing is achieved by diffusion. However, since the depth and width dimensions of the microchannel are small, the

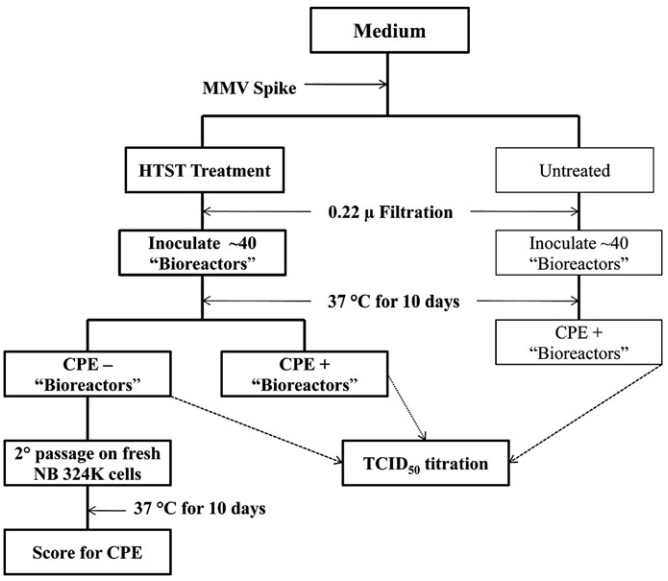

Fig. 1. Experimental schematics. LM 7105 culture medium was spiked with MMV to a final concentration of either 100 $TCID_{50}$/ml or 10 $TCID_{50}$/ml. The spiked LM7105 culture medium was then subjected to HTST treatment. The HTST treated LM7105 or control LM7105 were used to inoculate approximately 40 "bioreactors" (T_{25} flask). The inoculated "bioreactors" were incubated at 37 °C for 10 days and examined under a conventional microscope for CPE. Cell lysates from every CPE negative "bioreactors" were used to inoculate fresh NB 324 K cells.