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Schmallenberg virus, an emerging viral pathogen of cattle and sheep and a potential contaminant of raw materials, is detectable by classical in-vitro adventitious virus assays

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ABSTRACT

Emerging viruses, as potential contaminants of raw materials used in the manufacture of biologicals represent a challenge in the safety testing of biopharmaceutical products intended for human or veterinary use. Here, we report the challenge of an *in vitro* adventitious virus platform used in safety testing of biologicals, where a broad panel of detector cell lines was challenged to provide evidence that Schmallenberg virus is detectable by a classical reporting endpoint of cytopathic effect with Vero, BHK-21 and CHO-K1 detector cells, within typical *in vitro* assay timescales. We conclude that Schmallenberg virus is robustly detectable by classical *in vitro* viral biosafety assays.
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1. Introduction

Emerging viruses are a constant challenge to the safety testing of biopharmaceutical products intended for human or veterinary use. In 2011 a novel Orthobunyavirus was described as a pathogenic infectious agent of ruminants in Germany [1] and was named after the town in which it was first detected; Schmallenberg, in Hochsauerland, Germany. Orthobunyaviruses are enveloped viruses and possess segmented RNA genomes of negative polarity. Genetic analysis enabled the virus to be classified into the Simbu serogroup [1] most closely related to Sathuperi virus and Douglas virus [2]. Schmallenberg virus (SBV) is transmitted by arthropods of the *Culicoides* family [3–5]. Geographical dissemination of SBV was rapid through Europe during 2011–2013 and remains a concern for the livestock industry and the biopharmaceuticals industry. In adult ruminants, infection with SBV results in fever, diarrhoea and reduced milk yields. When infection occurs during gestation, SBV crosses the placenta [6] and can result in severe birth defects and still births. The risk to contamination of biological matrices used in production of vaccines and biologicals stems from the potential presence of viral contaminants in raw materials of animal origin. Indeed, where an animal is infected with SBV, the virus is known to be disseminated throughout the body and is detectable in several organs and fluids [7].

Emerging viruses circulating in livestock pose the risk of being present in raw materials such as viral field isolates for viral seed generation, primary cellular explants for cell line development, serum for manufacture, trypsin for cell culture and animal peptones for culture. Recent reports of the discovery of novel viruses such as a “HoBi”-like pestiviruses [8] and the adenovirus-dependent parvoviruses BPV3 and BPV4 in bovine serum [9] provide clear examples of previously unidentified viruses, and considerable attention is now paid to risk mitigation strategies for the control of viruses in the manufacturing process. In the case of SBV, vaccines or therapeutic products contaminated with the virus may have serious consequences for recipients of veterinary vaccines, yet where humans are the intended recipient, the low probability of a disease should not result in a relaxation of the stringency and rigour of testing. To date, there is no evidence that SBV produces productive infections in humans and it is generally believed that SBV poses little if any threat to healthy immunocompetent humans [10,11]. However, while it is not possible to fully eliminate the risk of adventitious viruses in raw materials, an appropriate degree of assurance of freedom from extraneous agents and adventitious viruses should underpin the qualification of raw

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materials using suitable testing technologies for the detection of those viruses, and implementing new tests, where required, to ensure robust testing.

Several molecular methods for the detection of SBV have been reported [7,12,13]. These methodologies are well suited for SBV detection and invaluable for diagnostic purposes and disease control [14]. Considering biosafety testing of raw materials and biologicals, assays based on polymerase chain reaction technology (PCR) are often made available for viruses not detectable by general *in vitro* tests.

SBV has been shown to replicate and induce cytopathic effects (CPE) in several cell lines, including BHK-21 and Vero cells, indicating that a classical *in vitro* assay may be suitable for detection, but formal evidence of this remains lacking. The aim of this study was to evaluate the *in vitro* adventitious virus assay platform, where detector cell lines which are most frequently used in biosafety testing for raw materials and biopharmaceuticals are challenged with SBV to assess the sensitivity and timely manner of detection of SBV within *in vitro* testing assays compliant with regulatory authorities' guidelines and recommendations. To this end, the following cell lines were tested for their susceptibility to SBV: MRC-5, CHO-K1, 324K, Vero CCL-81, bovine turbinate (BT), Madin-Darby bovine kidney (MDBK), Primary bovine embryo kidney (PBEK) and foetal lamb kidney cells (FLK). MRC-5 and Vero cells are a diploid human cell line and simian kidney cell line respectively, as specified for the qualification of cell substrates and other raw materials for production of viral vaccines by the FDA [15] and Ph. Eur section 5.2.3 [16] and Ph. Eur section 5.2.4 [17]. 324K and CHO-K1 detector cells are also frequently utilised for testing under Ph. Eur section 2.6.16 [18]. BT, MDBK, PBEK and Vero detector cells are used for *in vitro* virus safety testing in accordance with CPMP [19] and CVMP [20] for the use of bovine serum and other raw materials for the production of vaccines and biopharmaceuticals for human and veterinary use. Vero and MDBK, with the addition of FLK cells, are commonly used detector cells for *in vitro* testing of raw materials of ovine and caprine origin or destination, and for final products for these target species. The duration of *in vitro* adventitious virus assays may vary, according to specific regulations and depending on the material tested and production stage; generally, *in vitro* tests are of 14, 21 or 28 days duration. We were, therefore, particularly interested in which detector cells elicited a clear CPE within 14 days where SBV was present.

2. Material and methods

2.1. Cells and virus

Vero (CCL-81), CHO-K1 (CCL-61), MRC-5 (CCL-171), MDBK (CCL-22) and BT (CRL-1390) cells were obtained from American Type Culture Collection (ATCC®). The human kidney 324K cell line was obtained from Yale University. Primary bovine embryo kidney (PBEK) cells were obtained from Benchmark Biolabs (Lincoln, USA); foetal lamb kidney (FLK) cells were obtained from the University of Glasgow, UK. MRC-5 cells were cultured in high glucose Dulbeco's modified eagle's medium (HG-DMEM, Gibco, Paisley, UK) supplemented with 10% FetalClone III serum (Hyclone) and 2 mM L-Glutamine. All other cells were cultured in HG-DMDM with 10% FCS (Gibco) and 2 mM L-Glutamine. SBV (isolate BH80/11-4) was kindly donated by the Friedrich-Löffler-Institut (FLI, Insel Riemes, Germany). The virus was isolated from bovine serum originating from Schmallenberg (North Rhine-Westphalia, Germany 2011), and initially passaged once through KC167 cells (derived from *Drosophila* sp) and five times through BHK-21 cells at the Friedrich-Loeffler-Institut. In our laboratory SBV was passaged a sixth time on BHK-21 cells to produce a virus stock. The infectious titre was determined to be 2.2×10^4 $TCID_{50}$/ml on BHK-21 cells. It was viewed as important to retain a limited passage number, in order to ensure that the virus remained as close to the wild-type phenotype as possible, and to avoid using a lab-adapted strain for the purposes of establishing detection.

2.2. Detection of SBV by the in vitro adventitious virus assay platform

In order to determine which primary cells and cell lines are susceptible to SBV, detector cells were inoculated under assay conditions with a moderate viral inoculum of SBV. The bovine and ovine cell lines (MDBK, BT, FLK and BPEK) were seeded into T80 flasks at 5×10^4 cells/ml in 20 ml total volume of medium. The following day the subconfluent monolayers were inoculated with 2 ml assay medium (HG-DMEM with 10% FCS or 1% FCS, 1 mM none essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.4 μg/ml Amphotericin B and 50 μg/ml Gentamicin) or 2 ml assay medium containing 1200 $TCID_{50}$ of SBV. 324-K, CHO-K1, MRC-5 and Vero cells were seeded into T80 flasks at 5×10^4 cells/ml in 20 ml total volume of medium. The following day the subconfluent monolayers were inoculated with 3 ml assay medium or 3 ml assay medium containing 1200 $TCID_{50}$ of SBV. For MRC-5 cells the assay medium consisted of HG-DMEM with 10% FCS or 1% FetalClone III serum, 1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.4 μg/ml Amphotericin B and 50 μg/ml Gentamicin. For all other cells the assay medium was consistent with that used for the ovine and bovine cells.

For all cell lines and primary cell cultures, inocula were allowed to adsorb for 90 min at 37 °C then removed and each culture was re-fed with 20 ml of fresh assay medium. The cultures were observed regularly for CPE. PBEK, MDBK, BT, Vero and FLK cells were subcultured on days 8, 15, 21 and 28 of the assay, unless terminated due to progression of CPE. Subculture was performed by dissociation of the cells with trypsin/EDTA (Gibco) and re-seeding into fresh flasks at a ratio of 1 in 3 to 1 in 10, depending on cell type and confluence. 324K and MRC-5 cells were re-fed with fresh medium once per week, typically 3–4 days post-subculture and subcultured on days 8 and 15 of the assay to maintain cell health.

To demonstrate the level of sensitivity for SBV in our *in vitro* adventitious virus assay platform, all cell lines except MDBK and MRC-5 were challenged with inocula of SBV at 5 and 50 $TCID_{50}$. BHK-21 cells were also included as these had served to propagate the virus and were therefore expected to show clear susceptibility to SBV and produce robust CPE at the lower inoculum levels. The cells were seeded into 6 well plates at 5×10^4 cells/ml in 3 ml. On the following day the cells were inoculated with 5 or 50 $TCID_{50}$ of SBV in a volume of 0.5 ml. Each level of inoculum was assayed in quadruplicate. Following an adsorption period of 90 min at 37 °C, the inocula were removed and the cells fed with 3 ml of fresh assay medium. The cells were observed regularly for CPE and maintained as described above. For clarity, where the reports of CPE were observed, the days of culture taken to enable detection by report of CPE refer to the time post inoculation, where, for instance, an observation of CPE in FLK cells at day 12 corresponds to the fourth day of subculture one of the FLK cells.

3. Results

3.1. Detection of SBV in detector cell lines for raw materials and veterinary vaccine safety testing

Vero cells demonstrated substantial CPE one day after inoculation with 1200 $TCID_{50}$ of SBV (Table 1) where rounding of cells and rapid cell detachment was clearly visible (Fig. 1). Upon challenge

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