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The Removal of Viruses During the Purification of Equine Antisera using Filtration Aids Hyflo Super-Cel™ and Fulmont™ Super A

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Abstract. The manufacturing process in Australia for equine antisera against various venoms/toxins is based primarily on ammonium sulphate precipitation of pepsin-digested IgG, whereby Fc and $F(ab')_2$ fragments are separated. The capacity of the process to remove non-enveloped and enveloped model viruses was assessed using a scaled-down process. Each virus was added to mid-process samples from equine plasma before the material was applied to Hyflo Super-Cel™ filtration followed by Fulmont™ Super A filtration. Samples were analysed pre- and post-filtration and the log clearance of the viruses calculated. The mean clearance factors for viral load of canine adenovirus type II (CAV_2), poliovirus type 1 (PV1), infectious bovine rhinotracheitis virus (IBR) and canine distemper virus (CDV) were 5·3 logs, 4·2 logs, 5·7 logs and 4·0 logs respectively. Clearance results as virus is adsorbed to the filtration aids which are removed from the process, thereby demonstrating improved viral safety of equine antisera produced by CSL.

Introduction

This study was undertaken to validate the equine antivenom/antitoxin process's capacity to inactivate or lower the titre of living virus(es) which might be present in hyperimmune equine plasma, the starting material for the antivenom/antitoxin against venomous snakes, spiders and toxins in Australia. The process is divided into steps, which were validated for viral inactivation/removal capacity, also known as viral clearance. The inactivation studies were conducted with four model viruses which are thought to be representative of a broad range of virus types which may be present in equine plasma. The viruses were selected to model potential contaminants.

Ammonium sulphate precipitation is a commonly used manufacturing technique to precipitate immunoglobulins from plasma. In the production process studied, pepsin is used to digest the immunoglobulin and the product, $F(ab')_2$, is separated from other proteinaceous components by filtration. Two separate components are used in the filtration process, to assist in removal of precipitated protein: Hyflo Super-Cel™ (diatomaceous earth) and Fulmont™ Super A (Fullers earth).

The filtration aids, Hyflo Super-Cel™ and Fulmont™ Super A, are used commonly in wine making to reduce turbidity,[1,2] in food technology applications[3,4] and in the water purification setting.[5–9] The capability of filtering aids to lower the levels of yeast and bacteria in wine preparations has been described,[10] as has their ability to remove viruses and endotoxins from water.[5–9] However, their utility as virus removal agents in a biopharmaceutical setting is not described in the literature.

This study seeks to assess virus clearance achieved using filtration aids Hyflo Super-Cel™ and Fulmont™ Super A during the purification of equine $F(ab')_2$ using an ammonium sulphate precipitation process. Several enveloped and non-enveloped viruses were used as surrogate markers of equine viruses which may be present in equine plasma. CDV† was used as a marker for the closely related Hendra virus (formerly called equine morbillivirus),

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†Abbreviations: CAV_2, canine adenovirus type II; PV1, poliovirus type 1; IBR, infectious bovine rhinotracheitis virus; CDV, canine distemper virus; TCID, tissue culture infectious dose; $\log_{10}$, viral loads are expressed as $\log_{10}$ of these values and are indicated as n $\log_{10}$; BS-C-1, African green monkey kidney cells; Vero, African green monkey kidney cells; MDCK, Madin Darby canine kidney; MDBK, Madin Darby bovine kidney.

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which has been shown to be transmitted from equides to humans. CAV_2 was used as a marker for equine adenoviruses. PV1 was used as a marker for any non-enveloped small virus that may be present in equine plasma. Finally, IBR was used as a model of equine herpes viruses. A herpes model is suggested in the EU Committee for Proprietary Medicinal Products (CPMP) Guidelines (268/95).[11] The guidelines stipulate that a process step must afford 4 logs of viral clearance to be considered effective.

Materials and methods

Hyflo Super-Cel™ (diatomaceous earth)

Hyflo Super-Cel™ (Celite Corporation, Lompoc, CA, U.S.A.) is an off white powder made of small plankton marine diatoms, which is used for many purposes as a filtration aid. Hyflo Super-Cel™ is approximately 90% SiO_2 with trace amounts of other metal oxides. The pH of the material is 10 and the specific gravity 2·3%.

Fulmont™ Super A (Fullers earth)

Fulmont™ Super A (Laporte Industries Ltd, Cheshire, U.K.) is a grey or greyish-white powder or granules consisting mainly of hydrous aluminium magnesium silicate, which is often used as a filtration aid. The pH of the Fulmont™ Super A falls between 3·5–4·1. The acidic activated clay that is Fulmont™ Super A is highly adsorptive, this capacity making it particularly suitable for removal of impurities such as phosphatides, trace metals, colouring pigments, breakdown product and oils and fats.

Eagle's minimal essential medium

Eagle's minimal essential medium (MEM) with non-essential amino acids, single strength, made by CSL Limited (Parkville, Australia), is used for all cell culture.

Foetal bovine serum

Foetal bovine serum (FBS), CSL Limited (Filtron) is used for all cell culture assays and has been gamma irradiated with 30 kiloGrays. FBS is stored frozen at -70°C or thawed for use at 2–8°C for up to 4 weeks.

Canine distemper virus (model of equine morbillivirus)

Both canine distemper virus and equine morbillivirus belong to the genus morbillivirus in the family *Paramyxoviridae*. CDV is an enveloped RNA virus with a pleomorphic/spherical morphology and a size ranging from 250–300 nm. The resistance to harsh physicochemical treatment of canine distemper is low. CDV (CSL strain 1934), was grown in African green monkey kidney (BS-C-1) cells. The titre of the CDV batch used (002, 5/98) was 6·9 ($\log_{10}TCID_{50}$/ml).

Infectious bovine rhinotracheitis virus (model of equine herpes virus)

Infectious bovine rhinotracheitis virus is a bovine herpes virus and is a member of the *Herpesviridae*. Both bovine and equine herpes are large enveloped DNA viruses. The virus is within the size range 150–200 nm, and is spherical in shape. The resistance to harsh physicochemical treatment is low, but it is a good model of large-enveloped DNA viruses. IBR (Strain V155), was grown in Madin Darby bovine kidney (MDBK) cells. The titre of the IBR batch used (001 4/98) was 8·0 ($\log_{10}TCID_{50}$/ml).

Canine adenovirus type II (model of human or equine adenoviruses)

Canine adenovirus type II is a member of the *Adenoviridae*. Adenoviruses are non-enveloped DNA viruses that are fairly small (60–70 nm) and generally very highly resistant to physicochemical treatments (e.g. $\leq$pH 3). CAV_2 (FDL strain) was grown in Madin Darby canine kidney (MDCK) cells. The titre of the CAV_2 batch used (001, 4/98) was 8·9 ($\log_{10}TCID_{50}$/ml).

Poliovirus type 1 (model of small non-enveloped RNA virus)

Poliovirus is a human virus which is a member of the *Picornaviridae*. As the name suggests it is a small RNA virus and it is non-enveloped. The virus is 28–30 nm in size, and is of medium resistance to physicochemical treatment, with significant resistance to low pH ($\leq$3), but with moderate sensitivity to heating. PV1 (strain Sabin 1), was grown in African green monkey kidney (Vero) cells. The titre of the PV1 batch used (002 5/98) was 9·1 ($\log_{10}TCID_{50}$/ml).

Cytotoxicity analysis

Cytotoxicity analysis with production samples was performed on cells used in viral assay to determine if overt toxicity or changes in cell growth is caused by the samples. Results were reported qualitatively and cytotoxicity was judged to be absent if the amount of rounded cells was similar to

the controls and inhibition of cell growth was not evident.

Virus quantitation assays

All viruses and cells were grown in MEM (CSL), containing 5% FBS (CSL) at 36°C and 5% CO_2, for 5 days. The culture supernatant was clarified from debris by centrifuging samples at 300 ***g*** for 10 min. Viral titres were determined in 96-well flat-bottomed tissue culture plates containing cells. Daily observations were made for cytopathic effects for 7 days. The same virus preparation was used for all experiments and was stored aliquoted at −70°C until thawing immediately prior to the experiments.

On all cell lines (BS-C-1, MDBK, MDCK and Vero) the production samples were cytotoxic to cells when 25 μl of sample was added neat to 100 μl of cells. To overcome this effect all samples were diluted 1 in 25 in assay medium (AM) prior to testing and the dilution factor is taken into account in all calculations. Pre-dilution of production sample 1 in 25 in assay medium (AM:MEM, 5% FBS, 1% glutamine, 2% HEPES, 1% sodium bicarbonate) was performed with dilution of sample 1 in 5 performed in the first well of the plate (25 μl into 100 μl of culture medium), then five-fold dilutions were performed across the plate, with tip changes between each well.

Production process

The purification process applied to equine plasma is used to purify $F(ab')_2$ fragments of immunoglobulin from other proteins in the mixture. Initially the immunoglobulin is digested with pepsin, the Fc and other protein components are then precipitated using ammonium sulphate. The filtration step of the process, that separates the precipitated protein, consists of addition of Hyflo Super-Cel followed by filtration. The filtrate then has Fulmont™ Super A added to it. At small-scale, the sample had the same concentration of all reagents added as in production-scale. Since production-scale batches are normally 250 l, linear scale-down of all reagents and quantities was performed on an in-process sample to mimic viral inactivation conditions during typical product manufacture. An in-process sample of 100 ml was collected from a typical production run just prior to the Hyflo Super-Cel™ filtration step. The 100 ml study sample was then subject to further processing (filtration) at small scale, with the same concentration of all reagents added in accordance with the production scale formulae. For the viral inactivation experiments, 10 ml volumes of this study sample were assessed at least twice for all viruses with a negative control. The 10 ml samples were each spiked with 1 ml of the appropriate virus. A scaled-down version of the production-scale filtration was performed. Following filtration, the virus sample was diluted 1 in 25 in cell culture assay medium and then diluted for assay. A reference virus sample was run in parallel to all experiments to ascertain whether the virus was of sufficient quality for each assay performed. Experiments had seven replicates within each assay to ensure statistical accuracy.

Results

Viruses used and design of viral inactivation experiments

When performing viral validation, at least one model chosen should contain RNA and another DNA. In addition, representatives of both non-enveloped and enveloped viruses should be included, and a small virus should be included if possible. As the equine antitoxin/antivenom process contains many solvent exposure steps, two non-enveloped viruses were included as models, i.e. PV1 and CAV_2, RNA and DNA viruses respectively, which are also small. Two enveloped viruses CDV, and IBR were selected, RNA and DNA viruses respectively. The substitution of non-equine viruses is acceptable since the chosen picornavirus and adenovirus strains represent families of virus that have similar physical virion structure as equine strains. Viral clearance factors are calculated using the formula for calculation described by the EU CPMP Guidelines (268/95).[11] The virus clearance factor, C, for an individual inactivation or removal step is given by the expression:

$$C = \log\{(V1 \times T1)/(V2 \times T2)\}.$$

Where, C = the clearance factor, V1 = volume of the starting material, T1 = concentration of virus in starting material, V2 = volume of material after the step, and T2 = concentration of virus after the step.

A mean of 5·3 logs was measured for CAV_2, or a minimum of 4·6 logs.

A mean of 4·2 logs was measured for PV1, or a minimum of 3·4 logs.

A mean of 5·7 logs was measured for IBR, or a minimum of 5·1 logs.

A mean of 4·0 logs was measured for CDV, or a minimum of 3·7 logs.

Table 1. Clearance of CAV_2 by combined Hyflo Super-Cel™/Fulmont™ Super A filtration*

Experiment	V1	T1	Pre-load	V2	T2	Post-load	Clearance factor
1	1	7·8	8·8	2·2	≤2·0†	≤4·2	≥4·6
2	1	8·8	9·8	2·2	≤2·0†	≤4·2	≥5·6
3	1	8·9	9·9	2·2	≤2·0†	≤4·2	≥5·7

*Values expressed as $n\log_{10}$ where n=value.
†Limit of detection of assay.

Table 2. Clearance of PV1 by combined Hyflo Super-Cel™/Fulmont™ Super A filtration*

Experiment	V1	T1	Pre-load	V2	T2	Post-load	Clearance factor
1	1	9·5	10·5	2·2	4·5	6·7	3·8
2	1	9·9	10·9	2·2	5·3	7·5	3·4
3	1	9·7	10·7	2·2	3·1	5·3	5·4

*Values expressed as $n\log_{10}$ where n=value.

Table 3. Clearance of IBR by combined Hyflo Super-Cel™/Fulmont™ Super A filtration*

Experiment	V1	T1	Pre-load	V2	T2	Post-load	Clearance factor
1	1	8·3	9·3	2·2	≤2·0†	≤4·2	≥5·1
2	1	9·4	10·4	2·2	≤2·0†	≤4·2	≥6·2
3	1	9·1	10·1	2·2	≤2·0†	≤4·2	≥5·9

*Values expressed as $n\log_{10}$ where n=value.
†Limit of detection of assay.

Table 4. Clearance of CDV by combined Hyflo Super-Cel™/Fulmont™ Super A filtration*

Experiment	V1	T1	Pre-load	V2	T2	Post-load	Clearance factor
1	1	6·9	7·9	2·2	≤2·0†	≤4·2	≥3·7
2	1	7·3	8·3	2·2	≤2·0†	≤4·2	≥4·1
3	1	7·4	8·4	2·2	≤2·0†	≤4·2	≥4·2

*Values expressed as $n\log_{10}$ where n=value.
†Limit of detection of assay.

The filtration step (Hyflo Super-Cel™/Fulmont™ Super A) is effective at clearing on average 5·3 logs of CAV_2, 4·2 logs PV1, 5·7 logs IBR and 4·0 logs of CDV. This suggests that the filtration aids are effective at non-specifically removing viruses from a process irrespective of the physical properties of the virus.

Discussion

The use of filtration aids to improve the purity and to reduce levels of impurity in various samples is not a new concept.[1,3,7] The use of filtration aids may be useful in a biopharmaceutical setting if large sediments and viruses need to be removed from the product stream.

Appropriate selection of viruses for use in validation of processes is fundamental. Hendra (formerly known as equine morbillivirus) is a recently discovered virus, of particular interest as it has been transmitted from horse to human where it proved fatal.[12,13] The likelihood of Hendra presence in the herd of horses used to generate hyperimmune plasma is low, as they are routinely screened, however, inclusion of a model of such a virus is useful as it provides information about the worst case possibility of viral presence. A similar approach was taken in selection of the other three viruses.

In this study, care was taken to ensure production samples were processed at the small-scale using the same time limits and using the same reagents that are used at the large-scale. A 9% challenge of virus was added to the sample just prior to any processing of the sample. A 9% spike was chosen as this is below the accepted limit of virus that is satisfactorily measured without interfering with the properties of the starting sample (EU CPMP, 268/95).[11]

The filtration aids, Hyflo Super-Cel™ and Fulmont™ Super A proved effective in clearing significant levels of all viruses studied from the production process. These findings are similar to those in the literature which suggest that diatomaceous earth and Fullers earth provide a matrix for virus adsorption.[5–8] These filtration aids have also been shown to be useful in removing other types of microbial contamination including endotoxin and yeasts.[5,10]

Viruses have been shown to interact electrostatically with the filtration aids used in this study,[6,8,14] which can be controlled by maintaining pH, whereas hydrophobic interactions can be difficult to control. As well as electrostatic adsorption it is possible that virus may bind to Hyflo Super-Cel™ in ''clear pores'' in fragments of diatoms which are visible by scanning electron micrographs[8] or due to some level of mechanical straining. Electrostatic charge is also important in endotoxin removal,[5] and endotoxin removal was shown to be highly effective in the pH range 4·0 to 7·5, which is the same as that used by CSL.

Others have attempted to produce flow-through systems that are capable of processing large volumes while maintaining viral clearance capacity with limited success.[15,16] In our system large flow-through without clogging has not been a problem.

In a water filtration study[8] placing identical filters serially significantly improved virus adsorption, like this study the filtration is performed serially but in addition the filtration aid is changed (i.e. Super-Cel™, then Fulmont™), which also improves the clearance capacity overall.

The viral clearance capacity of Hyflo Super-Cel™ and Fulmont™ Super A was revealed during viral clearance validation of a pre-existing method where filtration aids were used in the process to help separate the precipitated Fc fragments and other precipitates from the $F(ab')_2$ product. It may be that other manufacturers use similar filtration processes without knowledge of their viral clearance capacity, and hence have not reported the effect. This study was designed to validate a pre-existing method, therefore it is limited in that different grades and types of filtration aids have not been investigated.

The presence of Hyflo Super-Cel™ and Fulmont™ Super A in the production process itself significantly improves the purity of the proteinaceous $F(ab')_2$ components in the mixture. The level of purification produced by the filtration aids is a very useful mid-stream step that was not originally introduced into the process for its viral removal capacity.

The overall viral clearance afforded by the equine antisera process was shown to be on average 5·3 logs of CAV_2, 4·2 logs PV1, 5·7 logs IBR and 4·0 logs of CDV. This level of viral clearance is at the significant 4 log level expected from a mid-process step by regulators.[11]

The Hyflo Super-Cel™ and Fulmont™ Super A filtration step in the equine antisera production process contributes significantly to the viral safety of the final product. The non-specific nature of adsorption of all viral types by Hyflo Super-Cel™ and Fulmont™ Super A, is excellent for reliance as a viral removal step; the two agents have differing physical properties, so the step is less likely to fail. The filtration step complements other process steps, including, for example, γ-irradiation of the starting material pepsin, recognized for viral clearance capacity, which combine to improve significantly the viral safety of equine antisera produced by CSL.

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