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ORIGINAL RESEARCH ARTICLE

Biological Safety of a Highly Purified 10% Liquid Intravenous Immunoglobulin Preparation from Human Plasma

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Abstract

Background A highly purified 10% liquid intravenous immunoglobulin, IQYMUNE®, has been developed using an innovative manufacturing process including an affinity chromatography step for the removal of anti-A and anti-B hemagglutinins.

Objectives The pathogen (viruses and prions) clearance efficacy of the manufacturing process and its robustness for critical steps were investigated.

Methods The manufacturing process of IQYMUNE® includes two dedicated complementary virus reduction steps: solvent/detergent (S/D) treatment and 20 nm nanofiltration as well as two contributing steps, namely caprylic acid fractionation and anion-exchange chromatography. The clearance capacity and robustness of these steps were evaluated with a wide range of viruses (enveloped and non-enveloped) and with a model of human transmissible spongiform encephalopathies (TSEs).

Results The IQYMUNE® manufacturing process demonstrated a high and robust virus removal capacity with global reduction factors (RFs) of relevant and model viruses: $\geq$14.8 $\log_{10}$ for human immunodeficiency virus type 1 (HIV-1), $\geq$16.9 $\log_{10}$ for bovine viral diarrhoea virus (BVDV)/Sindbis virus, $\geq$15.7 $\log_{10}$ for pseudorabies virus (PRV), $\geq$12.8 $\log_{10}$ for encephalomyocarditis virus (EMCV) and 11.0 $\log_{10}$ for porcine parvovirus (PPV). The process also exhibited a high removal capacity for the TSE agent with an overall RF of $\geq$12.9 $\log_{10}$ due to the complementary actions of the caprylic acid fractionation, anion-exchange chromatography and nanofiltration steps.

Conclusion Data from virus and prion clearance studies fully support the high safety profile of IQYMUNE®, with a minimal reduction of 11 $\log_{10}$ for the smallest and most resistant non-enveloped virus, PPV, and more than 12 $\log_{10}$ for the TSE agent.

Key Points

Dedicated steps as well as contributive steps to virus and prion clearance are required for plasma-derived medicinal products.

The manufacturing process of IQYMUNE® demonstrated very high capacity of inactivation and/or removal of viruses and prion.

1 Introduction

Plasma-derived medicinal products (PDMPs) play an important role in human medicine today with a number of purified proteins of major therapeutic interest treating several severe pathologies. Among these, intravenous immunoglobulins (IVIGs) represent the main product derived from plasma fractionation, and have an increasing need worldwide both in replacement therapy in primary and secondary immunodeficiencies and in an

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immunomodulation setting. Indeed, a number of autoimmune diseases, mainly neurologic, are successfully treated with IVIGs [1–4]. IQYMUNE® is produced by a manufacturing process based on caprylic acid fractionation and chromatography steps to achieve a high level of purity and safety while maintaining high yields of production, a critical point in a context of increasing demand. Of paramount importance for plasma-derived products is the assurance of product safety with regard to potential contamination by blood-transmissible pathogens such as viruses and prions, the causative agents of Creutzfeldt-Jakob disease (CJD) and its subsequently identified variant (vCJD). The safety of IQYMUNE® relies on (1) the quality of the starting material, human plasma, ensured by the careful selection of donors and screening of the plasma for viruses; and (2) the ability of the manufacturing process to remove and/or inactivate a wide range of known and potentially emerging viruses as well as ensuring the removal of prions [5, 6]. Validation of key biological safety process steps is essential in order to assess the virus/prion removal capacity with respect to these agents [7, 8]. The robustness of critical parameters for dedicated safety steps also needs to be evaluated to ensure the consistent, robust inactivation/removal across production runs.

We report on the validation of the virus removal and/or inactivation capacity and prion removal by the manufacturing process of IQYMUNE®. The contribution of four steps to virus clearance and three steps to prion clearance have been validated—20 nm nanofiltration, caprylic acid fractionation and anion-exchange chromatography for viruses and prions, and solvent/detergent (S/D) treatment for viruses—providing an optimum safety profile for IQYMUNE®. Results of robustness studies conducted for the S/D treatment, 20 nm nanofiltration and caprylic acid fractionation steps are also provided.

2 Materials and Methods

2.1 Pathogens and Cells

The viruses and prion agents used in this study are listed in Table 1. A wide range of viruses and a 263 K scrapie hamster brain microsomal fraction were used. For the 263 K agent, clarified brain homogenate (BH) from infected animals was centrifuged at 10,000*g* for 10 min and the supernatant ultracentrifuged at 140,000*g* for 60 min as previously described [9, 10]. The pellet was then resuspended in 0.9% NaCl or 5% glucose solution and stored at –60 °C or lower until use. The cells used for virus titration and the assays performed are described in Table 1.

2.2 Evaluation of Virus Reduction

Studies were carried out at BioReliance in Stirling, Scotland. Prior to performing any virus infectivity assay, tests were performed to determine the first non-cytotoxic and/or non-interfering dilution. Viruses were quantified by end-point dilution in 96-well plates with the appropriate cell line by monitoring the cytopathic effect. Virus titres were calculated using the Spearman-Kärber formula [12] or Poisson distribution (when no virus is detected) and reported as tissue culture infectious dose per mL ($TCID_{50}$/mL) [12, 13]. In order to improve the limit of detection, large volume plating (LVP) (consisting of the inoculation of 96-well plate(s) with sample at first non-cytotoxic and non-interfering dilution) was also performed on process samples, which were expected to contain no or only a few virus particles.

2.3 Evaluation of Prion Reduction

Quantification of the transmissible spongiform encephalopathy (TSE) agent in process intermediates was achieved using both the Western Blot (WB) method and bioassay (in Golden Syrian hamsters). For WB, process samples were digested with proteinase K (PK), boiled in denaturation buffer and serially diluted five-fold before analysis. Optimal PK concentrations were assessed for each type of sample. Protease-resistant prion protein (PrP^{res}) signals were revealed using the anti-prion protein (PrP) antibody 3F4 or the alkaline phosphatase-labelled version of the 3F4 antibody. The titre of the sample analysed was determined by taking the $\log_{10}$ of the first dilution where no PrP^{res} (28 kDa) could be observed. For the bioassay, process samples were serially diluted ten-fold and inoculated to groups of six animals (one group per dilution). Animals were observed for at least 365 days. The number of animals with clinical signs allowed to calculate a titre ($\log_{10}$ infectious dose 50% per mL [ID_{50}/mL]) using the method of Spearman Kärber [12]. Prion studies were carried out at BioReliance in Glasgow and Stirling (Scotland) for spiking and WB experiments and at BioReliance in Rockville (MD, USA) for bioassays.

2.4 Manufacturing Process

The IgG purification process (see Fig. 1) is based on ethanolic fractionation and caprylic acid precipitation followed by filtration steps (Supradur 50P depth filtration and AKS 5 carbon filtration [PALL LIFE SCIENCES, Bad Kreuznach, Germany]). To inactivate enveloped viruses, the protein solution is then subjected to S/D treatment (0.3% [v/v] tri-n-butyl phosphate [TnBP] and 1% [w/v]

Table 1 Description of selected pathogens used in the virus and prion studies

Pathogen (strain)	HIV-1	BVDV	Sindbis	PRV	SV-40	EMCV	PPV	Prion
Relevant or model for	HIV-1, HIV-2, HTLV	HCV, West Nile virus, Chikungunya virus	HCV, Chikungunya virus, West Nile virus	Human or animal herpesvirus	Polyomavirus, resistant virus to inactivation	HAV	Human parvovirus B19	CJD agents
Family	*Retroviridae*	*Flaviviridae*	*Togaviridae*	*Herpesviridae*	*Polyomaviridae*	*Picornaviridae*	*Parvoviridae*	NA
Strain	IIIB	NADL	AR339	Duvaxyn/ Aujeszky	PA57	EMC/Florida	NADL-2	263 K scrapie
Genome	RNA	RNA	RNA	DNA	DNA	RNA	DNA	NA
Enveloped	Yes	Yes	Yes	Yes	No	No	No	NA
Size (nm)	80–100	40–60[a]	60–70	120–200	40–50	25–30	18–24	NA
Physical or chemical resistance	Low	Low	Low	Medium	Very high	Medium	Very high	Very high
Propagation system	MT4 cells	MDBK cells	BHK-21 cells	BHK-21 cells	Vero cells	Vero cells	PT-1 cells	263 K-infected hamster
Assay system	$TCID_{50}$ C8166 cells	$TCID_{50}$ MDBK cells	$TCID_{50}$ Vero cells	$TCID_{50}$ Vero cells	$TCID_{50}$ Vero cells	$TCID_{50}$ L929 cells	$TCID_{50}$ PK-13 cells	Western blot analysis or bioassay[b]

BHK baby hamster kidney, *BVDV* bovine viral diarrhoea virus, *CJD* Creutzfeldt-Jakob disease, *EMC* encephalomyocarditis, *EMCV* encephalomyocarditis virus, *HAV* hepatitis A virus, *HCV* hepatitis C virus, *HIV-1* human immunodeficiency virus type 1, *HIV-2* human immunodeficiency virus type 2, *HTLV* human T-lymphotropic virus, *MDBK* Madin-Darby bovine kidney, *NA* not applicable, *NADL* National Animal Disease Laboratory, *PK* porcine kidney epithelial, *PPV* porcine parvovirus, *PRV* pseudorabies virus, *PT* porcine testis, *SV-40* Simian virus 40, $TCID_{50}$ tissue culture infectious dose 50%

[a] Virus sizes according to CPMP/BWP/268/95 [7] (except BVDV, size is according to [11])

[b] In female Golden Syrian hamster

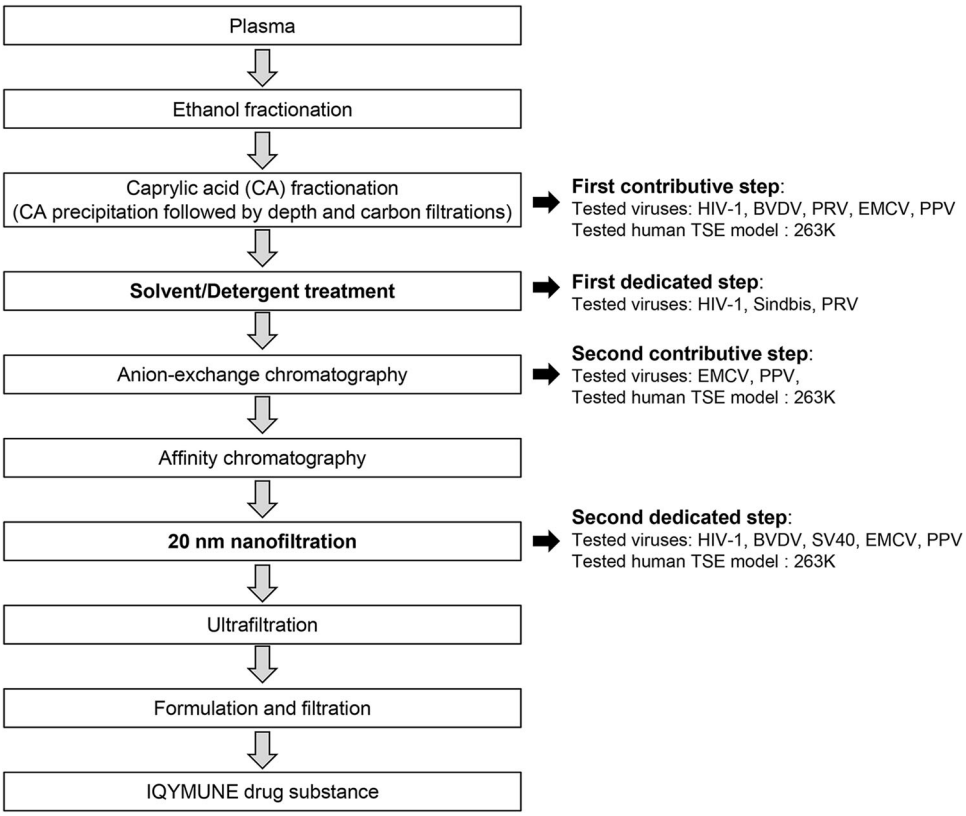

Fig. 1 IQYMUNE® manufacturing process: identification of virus inactivation/removal steps (dedicated steps in *bold*) and tested agents. *BVDV* bovine viral diarrhoea virus, *EMCV* encephalomyocarditis virus, *HIV-1* human immunodeficiency virus type 1, *PPV* porcine parvovirus, *PRV* pseudorabies virus, *SV-40* Simian virus 40, *TSE* transmissible spongiform encephalopathy

octoxinol for at least 6 h at 23 ± 3 °C). The IgG are then purified by a succession of two chromatography steps: an anion-exchange chromatography [Fractogel® EMD TMAE (MERCK MILLIPORE, Altdorf, Switzerland)] followed by an affinity chromatography [HyperCel™ IsoA/IsoB (PALL BIOSEPRA, Cergy Pontoise, France)]. The solution is then filtered through the sequence of 0.1 µm—Ultipor® VF DV50 (PALL LIFE SCIENCES, Ilfracombe, England)—Planova™ 20N (ASAHI KASEI, Nobeoka and Oita-shi, Japan) before the formulation stage.

2.5 Laboratory-Scale Pathogen Clearance Studies

Prior to undertaking the experiments, small-scale models of manufacturing processes were qualified for their relevance to industrial conditions. The critical operating parameters for each selected step are presented in Table 2. Process intermediates were spiked with selected viruses or the 263 K agent. For virus studies, the spiked material was prefiltered either on a 0.45 µm, 0.2 µm or 0.1 µm filter, depending on the size of the selected virus, to remove potential aggregates. Due to the heterogeneous

Table 2 Critical operating parameters for selected manufacturing steps

Step	Caprylic acid fractionation	S/D treatment	Anion-exchange chromatography	20 nm nanofiltration
Small-scale ratio	1/9000[a] and 1/14300[b]	1/10330	1/7100 or 1/7400	1/4000
Critical operating parameters	Caprylic acid to protein ratio (%)	Concentration of S/D reagents	Re-use resin	Protein load
	Volume of rinse for Supradur 50P filter	Temperature	Column height	Temperature
		Incubation duration		Pressure

S/D solvent/detergent

[a] For precipitation and Supradur 50P filtration

[b] For AKS 5 filtration

composition of the spiked material (resuspended precipitate), no filtration was performed before the caprylic acid fractionation evaluation. For prion studies, the spiked material was sonicated twice for 1–2 min before use. Before nanofiltration, the material was filtered with a 0.1 μm filter, as carried out in the manufacturing process. Unless otherwise specified, clearance studies were performed in duplicate runs.

2.6 Calculation of Reduction Factors

The clearance capacity of a step (reduction factor [RF]) was calculated by taking the ratio of the quantity of pathogen detected in the load sample over the quantity detected in the fraction collected after the step, expressed as a logarithmic value ($\log_{10}$). Three methods were used to calculate the final value of RF for a process step: (1) when no pathogen was detected in either of the two duplicate runs (both values are expressed with a '$\geq$'), the highest value of the duplicate RFs was selected; (2) when a pathogen was detected in only one of the two duplicate runs, the RF value of that run was selected; and (3) when a pathogen was detected in both duplicate runs, the mean value of the two RFs was chosen.

2.7 Solvent/Detergent Treatment

A mixture of solvent (TnBP) and detergent (octoxinol) was added to the spiked material and the solution was left with stirring during 6 h. The treatment was performed in industrial conditions (S/D 1: 0.3% [v/v] TnBP and 1% [w/v] octoxinol) at 25 °C and with lower concentrations of S/D (S/D 1/5: 0.06% [v/v] TnBP and 0.2% [w/v] octoxinol; S/D 1/100: 0.003% [v/v] TnBP and 0.01% [w/v] octoxinol) at 15 and 25 °C, respectively. As massive interference was observed in the bovine viral diarrhoea virus (BVDV) assay system in the presence of the load material (very few or no residual infectivity was recovered in the spiked material and filtered spiked material), Sindbis virus was selected as another model for HCV.

2.8 20 nm Nanofiltration

Clearance studies were evaluated under industrial conditions with the viruses and prions indicated in Fig. 1. Porcine parvovirus (PPV) was selected as the model virus for robustness studies, evaluating the effect of single or combined parameters (protein load, temperature and pressure). RF were calculated using the viral loads measured before and after the Planova™ 20N filter (ASAHI KASEI BIOPROCESS, Brussels, Belgium). Hold controls were used to distinguish between inactivation due to the low pH and removal by filtration. After nanofiltration, the integrity of the filter was verified according to the manufacturer instructions. Backflush samples were titrated to confirm the virus retention on the 20N filter.

2.9 Caprylic Acid Fractionation

For the validation of the caprylic acid fractionation step, the spiked material was adjusted to proper pH (pH 4.8) and a load sample taken for titration. Caprylic acid and filter aid were then added and following a defined contact time (at least 60 min), the solution was filtered through the Supradur 50 P filter (PALL France, Saint-Germain-en-Laye, France). The filtrate and rinse fractions were pooled (intermediate filtrate). Industrial conditions were investigated with viruses and prions as shown in Fig. 1. The AKS 5 filter (PALL France, Saint-Germain-en-Laye, France) was evaluated only with encephalomyocarditis virus (EMCV), PPV and prions. The filtrate and rinse fractions of the AKS 5 filter were collected and pooled (final filtrate). Robustness conditions were tested with PPV to investigate the effect of a lower and upper caprylic acid to protein ratio (90 and 110% of the target value) and extra rinse of the Supradur 50 P filter. The retentate of the Supradur 50 P filter was also titrated to assess the mechanism of virus clearance. RF were calculated for the Supradur 50 P filter alone (with the extra rinse) and coupled to the AKS 5 filter. Hold samples were collected to evaluate the effect of the time and of the pH of the matrix.

2.10 Anion-Exchange Chromatography

The anion-exchange chromatography [Fractogel® EMD TMAE (MERCK MILLIPORE, Altdorf, Switzerland)] was challenged with the spiked product subjected to S/D treatment to reproduce the chemical environment for the chromatography. This sample was then adjusted to the proper pH (pH 9) and conductivity before loading onto the column. The parameters tested consisted of lower and upper gel bed heights (55 and 110% of the target value) and gel aging by repeated use. The RF was calculated from the virus amounts in the eluate and load fractions.

2.11 Sanitisation Procedure Used for Anion-Exchange and Affinity Chromatography Steps

The sanitisation protocols of the anion-exchange (1 M NaOH/1 M NaCl for 60 min at room temperature [RT]) and affinity (1 M NaOH for 30 min at RT) chromatography columns were investigated using a carryover protocol. The column was loaded with a virus-spiked in-process fraction and the complete chromatography cycle (including regeneration and sanitisation steps) was performed. A second chromatography cycle performed with a non-virus-spiked

in-process fraction then provided a measure of the virus that remained attached to the column.

3 Results

3.1 Virus Clearance Studies

A summary of clearance data of the manufacturing process of IQYMUNE® is presented in Table 3. The data show a high viral reduction capacity with a minimum of 11 $\log_{10}$ for small non-enveloped resistant viruses (PPV) and higher RF for larger and/or enveloped viruses. The results are described for each individual step in more detail in Sects. 3.1.1–3.1.4, 3.2 and 3.3.

3.1.1 Solvent/Detergent Treatment

As expected, the S/D treatment performed for 6 h provided very effective inactivation for enveloped viruses with RF of $\geq$4.4 $\log_{10}$, $\geq$5.4 $\log_{10}$ and $\geq$4.3 $\log_{10}$ for HIV-1, Sindbis and pseudorabies virus (PRV), respectively. The inactivation kinetics, shown in Fig. 2, indicated that inactivation occurs within seconds ($\leq$1 min) of the treatment.

3.1.2 20 nm Nanofiltration

The sensitivity of HIV-1 to low pH, as shown by the lower virus titres in the load and hold samples, prevented us from measuring a RF with the 20 nm nanofilter for this virus. Due to its large size (120–200 nm), PRV was not considered relevant for a 20 nm filtration step. The RF measured with BVDV ($\geq$6.4 $\log_{10}$), a more resistant (<2 log of virus inactivation was only observed with BVDV) and smaller enveloped virus than HIV-1, was taken as the RF for HIV and PRV for this step. The nanofiltration was very effective for non-enveloped viruses, with RF of $\geq$4.8 $\log_{10}$, $\geq$5.9 $\log_{10}$ and 3.5 $\log_{10}$ for tested viruses SV-40, EMCV and PPV, respectively. The robustness of the 20 nm nanofiltration step was demonstrated using the smallest size virus, PPV. Higher pressure, higher protein load and lower temperature had no impact on this step with RF of 4.6 $\log_{10}$, 3.8 $\log_{10}$ and 4.0 $\log_{10}$, respectively. In contrast, at lower pressure, a trend toward lower efficacy was observed (RF range 2.7–3.8 $\log_{10}$; see Fig. 3).

3.1.3 Caprylic Acid Fractionation

For enveloped viruses, RF $\geq$4.0 $\log_{10}$, 5.1 $\log_{10}$ and $\geq$5.0 $\log_{10}$ for HIV-1, BVDV and PRV, respectively, were measured for the combined caprylic acid precipitation and Supradur 50 P (PALL France, Saint-Germain-en-Lay, France) filtration steps.

For non-enveloped viruses [caprylic acid precipitation followed by Supradur 50P and AKS 5 filtration steps (PALL France, Saint-Germain-en-Laye, France)], complete reduction of EMCV was evidenced with very high RF ($\geq$5.6 $\log_{10}$). No or very low level of infectivity was detected for EMCV in the Supradur 50 P filtrate and no infectivity was recovered in the AKS 5 filtrate (Table 4). Similar levels of PPV clearance were observed between intermediate and final filtrate fractions (3.9 $\log_{10}$ and 3.7 $\log_{10}$, respectively). The robustness of critical parameters (caprylic acid to protein ratio and extended rinsing applied to the Supradur 50 P filtration) was investigated with PPV and showed no significant impact in comparison to the industrial conditions (RF of 4.6 $\log_{10}$ and 3.8 $\log_{10}$ compared to 3.8 $\log_{10}$ under industrial conditions; see Table 4). No or very low levels of PPV were released from the Supradur 50 P filter despite extended rinsing (data not shown).

Table 3 Overall viral reduction capacity of the IQYMUNE® manufacturing process

	HIV-1	BVDV	PRV	SV-40	EMCV	PPV
Model for	HIV	HCV	Herpes DNA EV (HBV)	Highly resistant NEV	HAV	Parvovirus B19
Step						
Caprylic acid fractionation	$\geq$4.0	5.1	$\geq$5.0	NT	$\geq$5.6	3.7
S/D treatment	$\geq$4.4	$\geq$5.4[a]	$\geq$4.3	NA	NA	NA
Anion-exchange chromatography	NT	NT	NT	NT	1.3	3.8
20 nm nanofiltration	$\geq$6.4[b]	$\geq$6.4	$\geq$6.4[b]	$\geq$4.8	$\geq$5.9	3.5
Overall viral reduction capacity	$\geq$14.8	$\geq$16.9	$\geq$15.7	$\geq$4.8[c]	$\geq$12.8	11.0

BVDV bovine viral diarrhoea virus, *EMCV* encephalomyocarditis virus, *EV* enveloped virus, *HAV* hepatitis A virus, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *HIV-1* human immunodeficiency virus type 1, *NA* not applicable, *NEV* non-enveloped virus, *NT* not tested, *PPV* porcine parvovirus, *PRV* pseudorabies virus, *S/D* solvent/detergent, *SV-40* Simian virus 40

[a] Value from Sindbis virus (another model for HCV)

[b] Estimated reduction factor, based on the value measured for BVDV a smaller virus

[c] Only one step evaluated

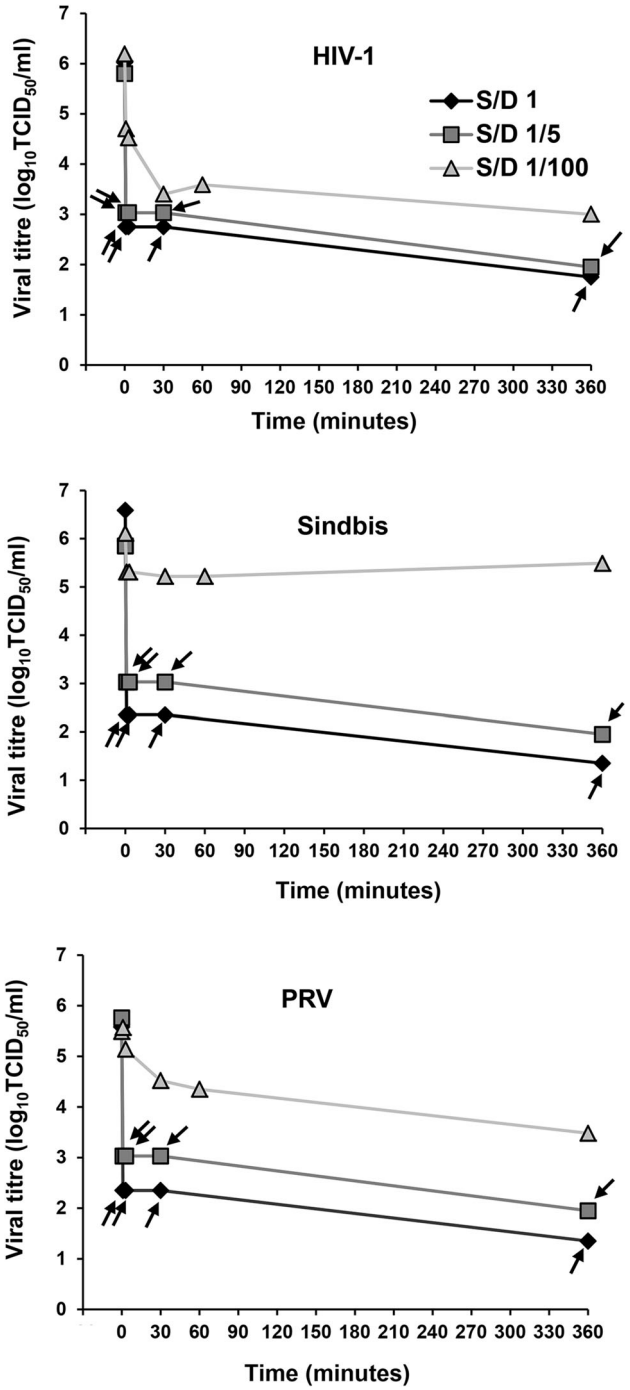

Fig. 2 Kinetics of inactivation of enveloped viruses at varying concentrations of solvent/detergent: S/D 1 industrial process conditions (0.3% tri-*n*-butyl phosphate/1% octoxinol), S/D 1/5 and S/D 1/100. Experiments were performed at 25 °C, except for condition SD 1/5 which was carried out at 15 °C. *Arrows* point out timepoints with no virus detected. Average of duplicate runs for S/D 1 and 1/5; single run for S/D 1/100. *HIV-1* human immunodeficiency virus type 1, *PRV* pseudorabies virus, *S/D* solvent/detergent, *$TCID_{50}$* tissue culture infectious dose 50%

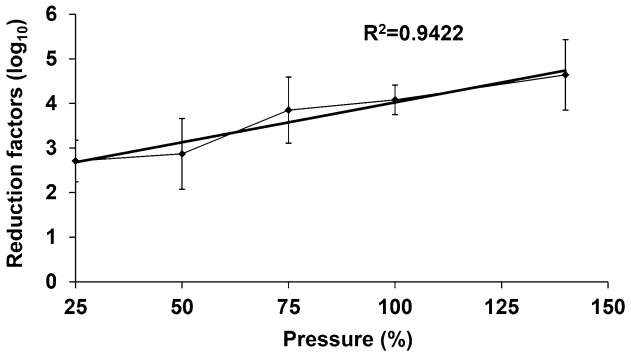

Fig. 3 Effect of pressure on the removal of porcine parvovirus by 20 nm nanofiltration. *PPV* porcine parvovirus

3.1.4 Anion-Exchange Chromatography

The anion-exchange chromatography step reduction of non-enveloped viruses was evaluated with EMCV and PPV: both low and high gel bed height extremes (55 and 110% of the target value) were tested with new gel and only the high gel bed height (110% of the target value) was tested with end-of-life gel (100 cycles). Low reduction was observed for EMCV (RF of 1.3 log_{10} and 2.1 log_{10} for the 55 and 110% conditions, respectively). Contrarily, the step showed substantial PPV removal under both conditions (RF of 5.6 log_{10} and 3.8 log_{10}; see Table 5). The virus removal was also evaluated with an end-of-life resin. No difference was observed between the two types of gel (EMCV: 1.6 log_{10}; PPV: $\geq$6.0 log_{10}; see Table 5).

3.2 Sanitisation Procedure Used for Anion-Exchange and Affinity Chromatography Steps

PPV, a highly resistant non-enveloped virus, was inactivated below detection for both gels, no PPV carryover was observed in the subsequent run (RF of $\geq$5.6 log_{10} and $\geq$6.6 log_{10} were observed for the anion-exchange and affinity chromatography, respectively). The data confirm the efficiency of the sanitisation treatments to both chromatographic columns.

3.3 Transmissible Spongiform Encephalopathy Clearance Studies

Data from TSE clearance studies are shown in Table 6. The caprylic acid fractionation step was shown to be very effective in prion reduction with an RF of $\geq$4.0 log_{10} by western blot, well-correlated to the bioassay ($\geq$5.2 log_{10}). For the anion-exchange chromatography step, RFs of $\geq$2.7 log_{10} (WB) and $\geq$3.9 log_{10} (bioassay) were demonstrated.

Table 4 Virus removal capacity of the caprylic acid fractionation step

	Manufacturing conditions					Robustness study	
	HIV-1	BVDV	PRV	EMCV	PPV	PPV	
Caprylic acid to protein ratio (%)	100	100	100	110	110	90	110
Virus reduction factor (log_{10})							
Supradur 50 P filtrate	≥4.0	5.1	≥5.0	6.1	3.9	3.8	3.3
Supradur 50 P filtrate with extra rinse	NT	NT	NT	NT	NT	3.8	3.3
Final (AKS 5) filtrate	NT	NT	NT	≥ 5.6	3.7	4.6	3.8

BVDV bovine viral diarrhoea virus, *EMCV* encephalomyocarditis virus, *HIV-1* human immunodeficiency virus type 1, *NT* not tested, *PPV* porcine parvovirus, *PRV* pseudorabies virus

Table 5 Virus removal capacity of the anion-exchange chromatography step

Column height (% of target value)	Cycle	Reduction factor (log_{10})	
		EMCV	PPV
55	0	1.3	5.6
110	0	2.1	3.8
110	100	1.6	≥6.0

EMCV encephalomyocarditis virus, *PPV* porcine parvovirus

Concerning the 20 nm nanofiltration step, no scrapie-associated prion protein (PrP^{Sc}) (RF ≥3.8 log_{10}) was detected after passing through the whole filtering sequence (0.1 µm—DV50—Planova™ 20N). The overall prion reduction capacity for the IQYMUNE® product is higher than 12.9 log_{10}.

4 Discussion

Amongst the different preventive measures against the risk of transmission of pathogens for PDMPs, some are related to the starting material, such as the selection of donors, testing of donations and testing of plasma pools. In addition, the manufacturing processes, which include efficient inactivation and/or removal techniques, contribute greatly to the biological safety of PDMPs. This article describes the virus and prion clearance evaluation for four steps of the manufacturing process of IQYMUNE®. The process includes two orthogonal-specific virus inactivation/removal steps—S/D treatment and 20 nm nanofiltration—as well as two steps with inherent virus/prion reduction capacity [13–17]—caprylic acid fractionation and anion-exchange chromatography.

S/D treatment is well-recognised as a highly efficient method for mitigating the risk of transmission of enveloped viruses by plasma products [18, 19]. In the IQYMUNE® process, the S/D treatment performed under standard operating conditions (0.3% TnBP and 1% octoxinol for 6 h at 23 ± 3 °C) resulted in complete inactivation of the three tested enveloped viruses within 1 min. This is in agreement with its initial description by the New York Blood Center and recent literature [19, 20]. The critical parameter of the S/D treatment is the concentration of the inactivating reagents, while other parameters such as protein content, pH and temperature have been described to have little or no impact on virus inactivation [19]. The robustness of the S/D treatment was substantiated by the immediate inactivation of the same viruses under conditions of lower S/D concentrations (1/5) and temperature (15 °C). For HIV-1, the inactivation kinetics remained similar even at 1/100th S/D concentration, confirming the high sensitivity of this virus to S/D exposure.

Validation studies with various production processes around the globe have confirmed that nanofiltration on small pore size filters is a reliable and robust method/technique for removing viruses and prions (for review see Burnouf et al. [21]). It can remove all types of viruses, including smaller and more resistant non-enveloped viruses. This technique is therefore complementary to the S/D treatment that inactivates only enveloped viruses. The nanofiltration step in the manufacturing process of

Table 6 Transmissible spongiform encephalopathy reduction capacity of the IQYMUNE® manufacturing process

Step	Western blot	Bioassay
Caprylic acid fractionation (precipitation followed by depth filtration)	≥4.0	≥5.2
Anion-exchange chromatography	≥2.7	≥3.9
20 nm nanofiltration	≥3.8	Not tested
Overall TSE reduction capacity	≥12.9	

TSE transmissible spongiform encephalopathy

IQYMUNE® was effective in removing all tested viruses, including PPV, a model of human B19 parvovirus. The RF for this virus, the smallest in the series (18–24 nm), is 3.5 $\log_{10}$. For the other two non-enveloped viruses investigated, complete removal was observed (RF $\geq$4.8 $\log_{10}$ for SV-40 and $\geq$5.9 $\log_{10}$ for EMCV) as anticipated. Concerning enveloped viruses, a $\geq$6.4 $\log_{10}$ removal was evidenced for BVDV. Due to the sensitivity of HIV-1 and PRV to the operating conditions (low pH), it was not possible to measure the removal capacity of this step for these viruses. However, the size exclusion mechanism of nanofiltration, as well as the data confirming significant removal of smaller viruses presented in this report is clearly in favour of the elimination of these larger viruses by this step. Moreover, the negligible impact of the robustness conditions (higher pressure, higher protein load and lower temperature) on the 20 nm nanofiltration performance observed with PPV reinforces the assurance that this step is highly efficient for virus removal. In contrast, reducing the pressure seemed to impact the nanofiltration removal since a tendency toward smaller RF was observed at lower pressures. For the pressure, the robustness studies lead, therefore, to a proven acceptable range of 0.6–1.1 bar.

Regarding the caprylic acid fractionation step, high RFs for enveloped viruses were obtained immediately after the precipitation and subsequent depth filtration step: $\geq$4.0 $\log_{10}$, 5.1 $\log_{10}$ and $\geq$5.0 $\log_{10}$ for HIV-1, BVDV and PRV, respectively. No virus was recovered in the precipitate (data not shown), indicating the viruses were inactivated during this step, as previously reported [15, 16, 22]. This step was also very effective in clearing non-enveloped viruses with RF of $\geq$5.6 $\log_{10}$ for EMCV and 3.7 $\log_{10}$ for PPV. For EMCV, clearance was almost complete (residual infectivity detected in one of the two runs) at the Supradur 50 P step and therefore additional viral clearance by the AKS 5 filtration could not be evidenced. Similar levels of PPV infectivity were obtained in the intermediate and final filtrates, indicating that no further reduction was achieved by the AKS 5 filter. These findings are consistent with previous observations obtained under slightly different conditions with Reo-3, hepatitis A virus (HAV) and PPV [23]. Studies conducted to test critical parameters (upper and lower values of caprylic acid to protein ratio and extended rinsing of the Supradur 50 P filter) demonstrated the robustness of this step with the most challenging virus, PPV. No PPV inactivation was measured in the hold controls and nearly all PPV infectivity was recovered in the precipitate sample, indicating that the reduction capacity of the step could be attributed to virus removal.

Because the S/D step was positioned just before the anion-exchange column, it was then judged more pertinent to evaluate this chromatography step exclusively with non-enveloped viruses. EMCV and PPV, selected as model viruses for HAV and B19, were spiked in in-process fractions and submitted to S/D treatment, in order to simulate the environmental conditions encountered by potential viral contaminants originating from the upstream process. Chromatographic removal of viruses from PDMP has long been documented [24]. The anion-exchange chromatography step was evaluated for two gel bed heights and with new and used gels. The clearance capacities of this anion-exchange chromatography column were high for PPV (3.8 $\log_{10}$ and 5.6 $\log_{10}$) and relatively low but significant for EMCV (1.3 $\log_{10}$ and 2.1 $\log_{10}$). After loading, no or a very low level of virus infectivity was detected in the unbound and washing fractions before elution, indicating binding of the viruses to the gel. Following the column regeneration step, the initial amount of virus was recovered in the regeneration fraction for both viruses, which demonstrated that partitioning was the mechanism of virus clearance. Results of viral clearance studies also demonstrated that end-of-life resin behaves identically (if not better) than new resin with regard to EMCV and PPV clearance.

Chromatography columns are sometimes used repeatedly and it is imperative that regeneration and sanitisation procedures in place be investigated under industrially relevant conditions to validate the absence of risk of carryover of potential contaminants from one production batch to the next. In the case of IQYMUNE®, this was confirmed with both anion-exchange and affinity chromatography steps. The regeneration/sanitisation sequence, which includes for both resins a 1 M NaOH treatment, is known to be efficient and robust for virus inactivation [25, 26] and also reduces the prion risk as demonstrated recently by our group [27]. Based on prion removal data in the literature and in-house knowledge of the manufacturing process, three independent process steps with potential capacity to remove prions were identified in the IQYMUNE® manufacturing process: caprylic acid fractionation [23, 28], ion-exchange chromatography [17, 28, 29] and nanofiltration [28, 29–32]. The overall prion removal capacity was found to be more than 12.9 $\log_{10}$, largely in excess of what is needed, according to current risk assessment calculations [32–35]. Moreover, no residual prion infectivity or protein was detected in any of the three steps validated, indicating that the real removal capacity of the process is likely much higher.

5 Conclusion

The manufacturing process of IQYMUNE® contains two specific and efficient orthogonal viral clearance steps (S/D treatment and 20 nm nanofiltration) and two contributive

steps with substantial virus inactivation/removal capacity (caprylic acid fractionation and anion-exchange chromatography). The overall RF for non-enveloped viruses, which are more resistant to inactivation and/or elimination than enveloped viruses, was more than 11 $\log_{10}$, providing a clear assessment of the safety of this product for all viruses. The high safety profile of this product also applies to prion clearance. Moreover, the validated regeneration/sanitisation procedures for reused chromatography resins also contribute to the safety of IQYMUNE®. Taken together, the data provided here offers thorough assurance of the high safety margin of the IQYMUNE® liquid IVIG product.

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Compliance with Ethical Standards

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Conflicts of interest The authors (CG, SS, SB, AB, CD, BY, PP, MO, LB, SC and BF) declare that they are employees of LFB, a pharmaceutical company that manufactures and commercialises IQYMUNE. CJ-K was employed by LFB at the time the experiments were carried out and the manuscript was prepared.

Ethical approval Viral and prion clearance studies were carried out in accordance with the principles of Good Laboratory Practice (as required by international regulatory guidelines) with a specialised Contract Research Organisation. Applicable international, national and/or institutional guidelines for the care and use of animals were followed at the place where animal studies were conducted.

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