

## Identification of Trimeric Peptides That Bind Porcine Parvovirus from Mixtures Containing Human Blood Plasma

Caryn L. Heldt,<sup>†</sup> Patrick V. Gurget,<sup>†,‡</sup> Lee-Ann Jaykus,<sup>§</sup> and Ruben G. Carbonell<sup>\*,†</sup>

Department of Chemical and Biomolecular Engineering and Department of Food, Bioprocessing and Nutrition Science, North Carolina State University, Raleigh, North Carolina 27695, and ProMetic Life Sciences, Montreal, Canada

Virus contamination in human therapeutics is of growing concern as more therapeutic products from animal or human sources come into the market. All biopharmaceutical processes are required to have at least two distinct viral clearance steps to remove viruses. Most of these steps work well for enveloped viruses and large viruses, whether enveloped or not. That leaves a class of small non-enveloped viruses, like parvoviruses and hepatitis A, which are not easily removed by these typical steps. In this study, we report the identification of trimeric peptides that bind specifically to porcine parvovirus (PPV) and their potential use to remove this virus from process solutions. All of the trimeric peptides isolated completely removed all detectable PPV from buffer in the first nine column volumes, corresponding to a clearance of 4.5–5.5 log of infectious virus. When the virus was spiked into a more complex matrix consisting of 7.5% human blood plasma, one of the trimers, WRW, was able to remove all detectable PPV in the first three column volumes, after which human blood plasma began to interfere with the binding of the virus to the peptide resin. These trimer resins removed considerably more virus than weak ion exchange resins. The results of this work indicate that small peptide ligand resins have the potential to be used in virus removal processes where removal of contaminating virus is necessary to ensure product safety.

### 1. Introduction

The removal of viruses, pathogenic microorganisms, and toxins is an important problem in the growing area of human therapeutics. Every year, more therapeutic products are produced from animal, human, or cell culture sources (1), and these sources contain an inherent risk of viral contamination. Therapeutic products from human blood plasma, antibodies, albumin, and factor VIII, just to name a few, could be infected with human immunodeficiency virus (HIV), hepatitis B, B19 virus (formally known as parvovirus B19), SARS coronavirus, or one or more emerging viruses that have yet to be identified (2). Cell cultures are often contaminated with retrovirus particles, belonging to the family of viruses that include HIV (3). Cell culture lines often used in the production of human antibodies may contain viruses such as murine parvovirus (MVM) or cytomegalovirus (4). While this contamination has been greatly reduced since the requirement of strict characterization of cell culture lines and the careful screening of human plasma donors (3), the risk of low levels of contamination still exists.

The FDA requires that any process that uses materials from living sources must have two viral clearance steps to lower the risk of contamination (5). These steps must demonstrate a distinct mechanism of virus clearance and achieve a minimum of 4 log removal, or 99.99%. There are two broad categories for viral clearance, inactivation and removal (6). Inactivation is often performed toward the beginning of the purification of a therapeutic and could involve a lowering of pH or heating of

the product. Both of these processes work well against enveloped viruses, but caution must be taken to not harm the desired protein product. Virus removal is often done at the end of a process and most commonly involves nanofiltration of the final product directly before formulation. Nanofiltration works well for viruses of large size. However, small viruses like parvoviruses are often of approximately the same size as the protein product, making it difficult to separate them by filtration (2). Complete removal of small non-enveloped viruses with 20 nm pore size filters has been accomplished, but there is significant fouling of these small-pored membranes that can lead to reductions in production rates (7, 8). Filtration of parvoviruses has been improved by flocculation of the virus particles through addition of cationic polymers (9) or amino acids (10), which allows the use of larger pore membranes that do not foul as quickly. Virus removal can also be accomplished using functionalized membrane surfaces. Quaternary amine groups have been attached to membrane surfaces to facilitate the removal of viruses through an ion exchange mechanism (11–13). Viral clearance validation may be achieved by conducting spiking experiments on normal process steps used in the purification of a therapeutic (i.e., chromatography columns, precipitation) (14), but care should be taken if a chromatography step is to be used concurrently as a viral clearance step and a protein purification step. If both the virus and the protein bind to the resin, it is possible for viruses to accumulate in the column. Without proper cleaning, the virus may elute from the column in subsequent batches and contaminate the therapeutic product (14).

Affinity adsorption is rarely used to remove viruses from process streams because the most common affinity ligands for viruses are antibodies. Antibodies are expensive to produce, often cannot withstand the harsh conditions required for the

\* To whom correspondence should be addressed. Ph: (919) 515-7054. Fax: (919) 515-5831. Email: ruben@ncsu.edu.

<sup>†</sup> Department of Chemical and Biomolecular Engineering.

<sup>‡</sup> ProMetic Life Sciences.

<sup>§</sup> Department of Food, Bioprocessing and Nutrition Science.

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cleaning of process equipment, and carry an inherent risk of being contaminated with viruses in their own right (3). However, affinity adsorption has been applied to the reduction of infectious prions from blood (15, 16), and a small peptide ligand has been found to remove staphylococcal enterotoxin B, a small toxin, from *E. coli* lysate (17). Small peptides are more robust than antibodies, and they are also less expensive and can be chemically synthesized, eliminating the risk of virus contamination. Peptides can handle the cycling of production and cleaning much better than antibodies, and by using small peptides, there is no three-dimensional structure that may be destroyed during processing. In this work, several trimeric peptides have been discovered that remove PPV from phosphate buffered saline containing as high as 7.5% human blood plasma. The peptides were found when a synthetic, solid-phase combinatorial library was screened for ligands that bind to porcine parvovirus (PPV). Solid-phase libraries allow screening directly on the chromatographic support that will be used as the separation media and have been successful in the discovery of many affinity peptide ligands (18–21). The discovered peptides can completely remove any detectable PPV from PBS and completely remove any detectable PPV from the first 3 column volumes when 7.5% human plasma is present. This work demonstrates that small peptides may offer a novel and effective method for removing viruses from complex mixtures.

## 2. Materials and Methods

**2.1. Materials.** Phosphate buffered saline (PBS) containing 0.01 M phosphate, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4 was purchased from Sigma (St. Louis, MO), and human blood plasma was a donation from the American Red Cross (Rockville, MD). Amino acids, phenol red, sodium carbonate, sodium phosphate, glucose, calcium chloride, sodium chloride, potassium chloride, and magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA). Eagles Minimum Essential Media (EMEM) was purchased from Quality Biologicals (Gaithersburg, MD). MEM vitamins, sterile PBS, trypsin, gentamicin, and glutamine, all for cell culture, were purchased from Invitrogen (Carlsbad, CA).

**2.2. Virus Propagation and Titration.** The porcine parvovirus (PPV) NADL-2 strain was titrated and propagated on porcine kidney (PK-13) cells, which were a gift from the American Red Cross (Rockville, MD). The PK-13 cells were maintained and the PPV propagated as described in Heldt et al. (22) using complete media, which consisted of EMEM supplemented with 2 mM glutamine, 1x gentamicin, and 10% non-heat-inactivated fetal calf serum (Hyclone, Logan, UT). Upon propagation of the virus, the cell culture flasks were frozen at  $-20^{\circ}\text{C}$  and thawed at room temperature. The cells were then scraped from the flask, and the solution was clarified by centrifugation at 3000 rpm for 10 min in an IEC Centra CL2 centrifuge (Thermo Electron, Waltham, MA). This solution was then stored at  $-80^{\circ}\text{C}$  until further use.

Radioactive PPV was prepared by metabolically incorporating a radiolabel during propagation by addition of  $^{35}\text{S}$  methionine and cysteine to the cell culture media. This was done by seeding the cells at  $6 \times 10^5$  cells per 75 cm<sup>2</sup> flask. The next day, the flask was infected with  $10^3$  MTT units of PPV in 1 mL of PBS. An MTT unit was defined as the concentration of virus where 50% of the cells were considered viable, as determined by the metabolic cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) as previously described (22). The MTT concentration can be observed optically and quantified by spectrophotometry. The flasks were placed in the incubator

at 5% CO<sub>2</sub>, 37 °C, and 100% humidity for 1 h. At this time, 5 mL of complete media was added to the flask, which was returned to the incubator. The next day, the media was removed from the flask and starvation media was added, which contained the same amino acids and essential nutrients as the EMEM, except for methionine. The cells were exposed to 5 mL of this starvation media for 1.5 h, and then EasyTag Protein Labeling mixture (Perkin-Elmer, Waltham, MA) was added to a final concentration of 50  $\mu\text{Ci}/\text{mL}$ . The cells were frozen at  $-20^{\circ}\text{C}$  when approximately 90% cytopathic effect was observed, usually after 4–5 days. Virus purification was done by CsCl gradient centrifugation, as described elsewhere (22), after which solutions were dialyzed against PBS for 3 days at 4 °C, stored at 4 °C, and used within 2 weeks.

All infectivity measurements were made using the MTT assay, which previously has been correlated to a TCID<sub>50</sub> (50% tissue culture infectious dose), a common method for the titration of infectious viruses (22).

**2.3. Primary Screening of Library.** A solid-phase combinatorial trimer library was made by Peptides International (Louisville, KY) using the divide-couple-recombine technique (23) on Toyopearl Amino 650 EC (Tosoh Bioscience, Montgomeryville, PA). The library had an alanine and 2 mini-PEG spacer arms [Toyopearl resin-Ala-(COCH<sub>2</sub>-(OCH<sub>2</sub>CH<sub>2</sub>)<sub>2</sub>-NH)<sub>2</sub>-X-X-X], where X is any naturally occurring amino acid except cysteine or methionine. The library was swelled in 20% methanol in DI water overnight and then buffer exchanged with PBS three times, with the last buffer exchange being overnight. Ten milligrams of dry library was taken and mixed with 50% human blood plasma in PBS for 1 h.  $^{35}\text{S}$ -Labeled PPV was added to the library at 10,000 CPM (about  $1 \times 10^{-3}$   $\mu\text{Ci}$ ) and allowed to equilibrate for about 1.5 h. The library was then placed in a disposable 10 mL fritted column (Bio-Rad Laboratories, Hercules, CA). The beads were washed with PBS followed by PBS containing an additional 1 M NaCl or KCl until no radioactivity could be detected from the wash. The beads were washed again in PBS to remove excess salt and then put into 20 mL of 1% low melt agarose (Bio-Rad Laboratories, Hercules, CA). This was poured onto a 160 mm  $\times$  180 mm GelBond (BioWhittaker Inc, Walkersville, MD) and allowed to dry for 3 days. Kodak BioMax MR Film (Kodak, Rochester, NY) was placed onto the dried gel for 10 days and developed with a Konica Medical Film Processor (Tokyo, Japan). A proprietary ligand found by the American Red Cross that binds to PPV (positive control) and a negative control of Amino 650M were used as markers to line up the film and the gel for visualization of radioactive beads. Positive beads were excised from the gel, boiled in water for 10 min each, and vortexed and the water changed for a total of three repeats to remove the agarose and the bound PPV from the beads. The beads then were sent to the Texas A&M Protein Laboratory (College Station, TX) for sequencing by Edman degradation.

**2.4. Chromatography to Verify Screening Results.** Peptide resins were synthesized on Toyopearl Amino 650M resin (Tosoh Bioscience, Montgomeryville, PA) by Peptides International (Louisville, KY) and were packed into disposable PIKSI columns (ProMetic Biosciences Ltd, Cambridge, England) with a total of 0.5 mL of settled resin in PBS per column. A Rainin (Oakland, CA) 8-channel peristaltic pump was used to add a solution of PPV supernatant in either PBS or 7.5% human blood plasma in PBS, at a rate of 0.1 mL/min. Ten 0.5 mL fractions were collected and tested for infectivity using the MTT assay and compared to the titer of the starting material before addition to the column.

**2.5. Acetylated Control.** The acetylated control was made by the acetylation of Toyopearl Amino 650M resin. About 50 mL of settled resin was added to a sintered glass funnel and allowed to drain. The resin was washed three times with 100 mL of 0.1 M NaOH. The resin was then washed with deionized water until the pH was below 8. The resin was placed into three separate 50 mL conical tubes and 30 mL of 0.5 M sodium acetate was added to each tube followed by end-over-end rotation for 10 min. A 100% excess of acetic anhydride (Riedel-Haen, Germany), which amounted to a total of 755  $\mu$ L, was dissolved into 3 mL of acetone, and 1.2 mL of the solution was added to each conical tube. The tubes were mixed for 2 h. The resin was then returned to the sintered glass funnel and washed three times with 100 mL of DI water, four times with 100 mL of 0.5 M NaOH, and finally at least 10 times with 100 mL of DI water, until the pH of the rinse was below 8. The acetylation was confirmed by taking 50  $\mu$ L of acetylated resin, 50  $\mu$ L of Toyopearl Amino 650M resin, and 50  $\mu$ L of DI water and adding two drops of ninhydrin reagent, 2% solution (Sigma, St. Louis, MO). After 1–2 min the resins were observed for color change; the acetylated resin and the DI water remained yellow, whereas the amino resin turned purple.

### 3. Results and Discussion

**3.1. Library Design.** Many hexameric peptide ligands have been found that can purify proteins (19, 24, 25) and toxins (17). Each of these peptide ligands were selected from a hexamer library, which contains over 34 million different combinations, when 18 of the 20 naturally occurring amino acids are used for library production. It would take a tremendous amount of effort to screen all of these sequences, and it is not necessary when purification is the intended use of the ligand. In general, a purification ligand is useful if it can bind over 90% of the target protein and is specific enough to produce an eluted protein that is 80–90% pure, but for virus removal, the goal is reduction of  $\geq 99.99\%$  of a virus, which is at femtomolar to picomolar concentrations. To improve the possibility of finding a ligand that can accomplish this, a trimeric library was designed and screened. This library contained only 5832 different sequences and could be screened many times over to compare different screening conditions. Further, by screening the entire library, there was a greater probability that one or more strongly binding ligands would be found, which would not necessarily occur with a hexamer library.

A spacer arm of two sets of two ethylene glycol units separated by a peptide bond (26) (designated AEEA-AEEA by Peptides International) was added to the library to increase the chances of finding a peptide ligand that bound to a conserved area on the virus surface. This spacer arm separated the peptide approximately 15 Å from the undisclosed spacer on the Toyopearl resin. It has also been shown that hydrophilic ethylene glycol does not bind proteins and makes a flexible yet inert spacer arm that allows movement of the ligand, improving binding (27–29). A surface map of PPV shows that there are canyons on the surface of the virus that are approximately 15 Å in depth (30), and so the spacer arm was designed to allow the peptide to reach into the depths of the canyons. For most non-enveloped viruses, it is accepted that the conserved amino acid sequences are located in the depth of these canyons because these are often the location of the receptor binding sites.

**3.2. Primary Screening.** The library beads were originally blocked with 50% human blood plasma before the virus was added. This blocked any of the peptides that had a high affinity for plasma proteins before the addition of PPV to the library.

**Table 1. Peptide Sequences Found from Primary Screening**

wash	sequence	
1 M NaCl	KNY	AKL
	WRW	KTF
	KKK	VWR
	KGK	RAA
	KYY	KRR
	FVV	
1 M KCl	FRH	KHR
	KAA	RTG
	RQQ	

After incubation with PPV, the beads were washed to remove any nonspecifically bound virus. One screening run was washed in 1 M NaCl and yielded a total of 24 positive beads from about 10,000; another screening run was washed in 1 M KCl and gave a total of 9 positive beads. Only those positive beads that had a large signal to size ratio (i.e., a small bead that gave a large signal), as determined by visual inspection, were chosen for sequencing. The results of the returned sequences are shown in Table 1. To better determine the significance of the different chemical groups, the amino acids were counted and compared to their probability of random occurrence (Table 2). A random occurrence was determined as the number of amino acids in the chemical group divided by the number of different amino acids in the library and then multiplied by the total number of amino acids found from the sequencing. For example, there are five different aliphatic amino acids, so the random number of aliphatic amino acids is calculated by dividing 5 amino acids by the 18 different amino acids used in this study, and the result multiplied by the 48 total amino acids in the 16 trimers found by screening. This gives the random occurrence of aliphatic amino acids of 13.3 indicated in Table 2. If the number of amino acids from a certain chemical group was close to the random occurrence number, then it was suspected that the chemical group was just randomly found and may have little to do with the binding of the virus. However, if the number was much higher than that expected to occur randomly, then that group was considered to be significant in the binding of the virus.

Table 2 shows the importance of basic amino acids in the binding of PPV and, to complement this, the lack of acidic groups associated with ligands found to bind to PPV. The results show that positive charges are important for the binding of virus. This is an expected result because canine parvovirus, a related parvovirus, has an isoelectric point of 5.3 (31), leaving it negatively charged at physiological pH, which corresponded to the conditions used in the screening studies. There was a random distribution of aromatic and aliphatic groups with seven sequences that contained aromatics and seven sequences that contained aliphatics. The sequences were then categorized into the following: those containing an aromatic amino acid, those containing an aliphatic amino acid, and those containing neither. Since all but one sequence contained a basic group, all sequences chosen for further screening contained a basic amino acid. From these categories, five sequences were chosen for additional screening using column chromatography: WRW and KYY, which contain aromatics; RAA, which contains an aliphatic; and KHR, which contains a histidine. Also, KKK and KRR were combined to form KRK, which contains basic residues.

**3.3. Column Chromatography.** The resins were packed into disposable columns and tested for breakthrough of PPV in the eluent using infectivity as the enumeration method. First, cell culture supernatant containing PPV was diluted with PBS to a final titer of about 6–7 log (MTT/mL) (approximately a 1:100

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Table 2. Chemical Characterization of Sequences

amino acid type	random <sup>a</sup>	actual <sup>b</sup>
aliphatic	13.3	11
cyclic imino	2.7	0
imidazole	2.7	2
basic	5.3	21
aromatic	8.0	9
hydroxy	5.3	2
amide	5.3	3
acidic	5.3	0

<sup>a</sup> The number of amino acids expected from a random distribution of amino acids in 16 trimeric peptides. <sup>b</sup> The number of each type of amino acid that was present in the 16 trimeric peptides.

to 1:10 dilution) and was filtered through a 0.22  $\mu\text{m}$  filter. Virus spiking studies should not be carried out at a dilution more concentrated than 1:10, as the virus solution, which contains contaminants from the cell culture from which it was created, may start to interfere with the virus clearance mechanism (3). However, the larger the initial viral load, the better the opportunity to validate a high degree of virus clearance. Virus clearance was calculated in accordance with the expression

$$\log \text{clearance} = -\log \left( \frac{\text{virus detected after clearance step}}{\text{total virus load}} \right) \quad (1)$$

The PPV breakthrough curves were determined by pumping virus-spiked solutions onto the peptide columns at 0.1 mL/min. Fractions equivalent to 1 column volume were collected, for a total of 10 column volumes, and the amount of PPV in the flowthrough fractions was determined. The results are plotted in Figure 1 as the percentage of the detectable PPV as a function of column volume. Presenting the results in terms of a percentage of the detectable clearance automatically accounts for the different initial virus titers of the various batches analyzed in these experiments.

In PBS, all of the resins were able to clear completely the detectable virus available in the solutions, as shown in Figure 1. This is in contrast to the amino resin control (with no peptides, which is considered a weak ion-exchange resin) that was not able to remove any significant amounts of PPV from PBS. Clearly, the peptides were responsible for the binding of the virus, and nonspecific binding to the resin surface was ruled out.

The small peptide resins have the ability to remove viruses from simple solutions such as water, suggesting potential application to water treatment. In fact, microfiltration is being considered as an alternative to chlorine treatment of water supplies (32), but the method suffers from many of the same difficulties as nanofiltration for therapeutic processes. Small viruses, like hepatitis A virus, which has a diameter of 27–32 nm (33), and norovirus, with a diameter of 30–40 nm (34), are able to pass through many nanofiltration and all microfiltration membranes. These viruses are shed in the feces of infected humans and are common contaminants of water supplies. Small peptide ligands theoretically could be placed on microfiltration membranes to improve virus removal without the need to use membranes of small pore size which often cause fouling (8) and may require high back pressures.

To challenge the peptides for their ability to remove PPV under therapeutic processing conditions, virus-spiked 7.5% human blood plasma was used. A 7.5% human blood plasma solution contains about 5 mg/mL of protein, which is approximately the amount of protein that can be found in a therapeutic protein product. There are two general viral clearance steps in a monoclonal antibody production process, i.e., a low

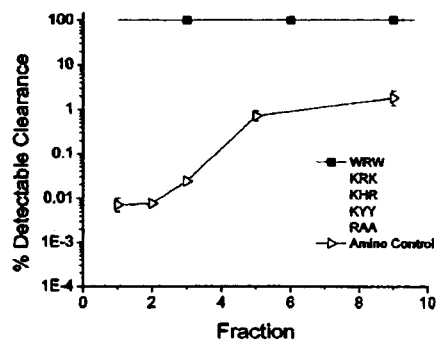


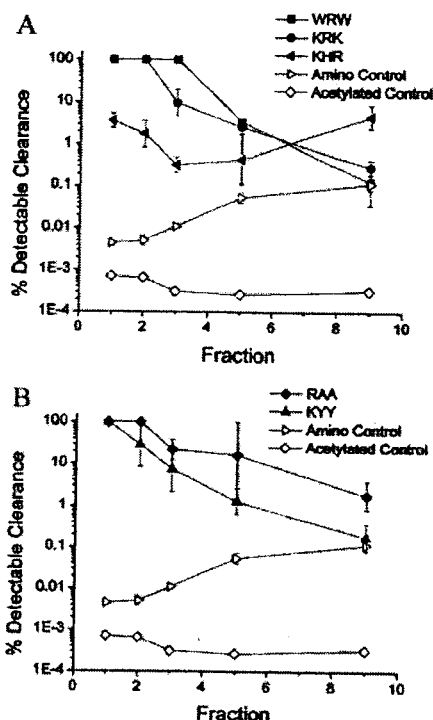
Figure 1. Binding of trimeric peptides to PPV in PBS. All ligands were able to bind 100% of the detectable PPV. The amino control, which is a weak ion-exchange resin, was able to clear less than 1% of the detectable PPV. Columns were run in duplicate, and the error bars represent the detectable clearance of each column.

pH step after cell harvest and a nanofiltration step before or during formulation (35). If effective, small peptides theoretically could be used on membranes on the nanofiltration step at the end of the process. All of the peptides were challenged with protein loads similar to those that would be found at this phase of the purification process.

Data on the removal of PPV from this complex mixture using the peptides are presented in Figure 2. All of the resins tested, except for KHR, were able to remove all of the detectable PPV in the first column volume. All of the resins had a breakthrough of PPV before the fifth column volume. It is believed that the proteins found in human blood plasma also began to nonspecifically bind to the peptides at this point, leaving less peptide available for specific binding to PPV. There may be a way to improve the selectivity of the virus over the plasma proteins by optimization of the chromatography process through changes in peptide density or buffer ionic strength.

The amino control resin had the ability to bind to some of the PPV and remove it from solutions. In the first two column volumes in PBS and 7.5% human blood plasma, the amino control achieved about 1 log clearance. This is not surprising, as anion exchange columns are often tested for their ability to clear viruses (35). It has been shown that a Q-Sepharose column was able to clear 3 log of PPV when loaded at pH 6.5 (36) and as high as 5 log of MVM when loaded in Tris buffer at pH 8.0 (14). This follows the trend that increasingly basic solutions will make the virus surface more negatively charged, which would cause increasingly stronger binding of the virus to an anion exchange column. The control in our experiments was a weak ion-exchange resin at pH 7.4, which showed clearance lower than that seen with the Q-Sepharose columns, as would be expected.

An increase in binding of virus over time was found with the amino control resin both in the presence and absence of human blood plasma (Figures 1 and 2). The PPV solution used in the experiments was cell culture supernatant that had been clarified by low-speed centrifugation and filtered through a 0.22  $\mu\text{m}$  filter, and so the solution contained cellular debris. Since the amino control is a weak ion-exchange resin, it is possible that the resin was binding the cellular debris, and the virus then bound to the debris on the resin, as many proteins in host cell proteins are known to be negatively charged and removed by anion exchange (35). Since the amino resin has a high positive charge density, it is also possible that these cellular proteins may denature onto the surface, as has been suspected in ion exchange purification (37, 38) and is known to happen when proteins adsorb to surfaces (39). Protein denaturation was also



**Figure 2.** Binding of trimeric peptides to PPV in 7.5% human blood plasma. WRW was able to bind 100% of the detectable PPV in the first 3 column volumes. After 9 column volumes, WRW was still able to bind as much as the amino control. The amino control and acetylated control are the same in each figure as a reference point for comparison of the different peptide resins. Columns were run in duplicate, and the error bars represent the detectable clearance of each column.

a possibility, as this was observed in the purification of tumor necrosis factor- $\alpha$  using an ion exchange column (37). The longer these host cell proteins are retained on the column, the greater the potential for protein denaturation, and this may provide different binding sites for the virus. Virus binding to host cell proteins is confirmed by the fact that when highly purified virus suspensions containing less than 100  $\mu\text{g}/\text{mL}$  of total protein are used in resin challenge, the amino control resin binds no more than 1 log of PPV even after 10 column volumes (data not shown). Viruses are also known to easily aggregate (40), and so the presence of denatured protein could become a new binding surface for the virus.

The trimer KHR was able to achieve 4 logs clearance in the first column volume but still left in solution 3 log (MTT/mL) of virus. This peptide column showed the same decrease in viral clearance in the first three column volumes seen with the other peptide resins, but in subsequent column volumes, it exhibited an increase in detectable clearance. This latter behavior was only seen in the amino control resin and not the other peptide resins. It is suspected that this resin may be causing denaturation of proteins in solution as discussed above, but this issue was not examined further. This resin was just discarded as one of the lead candidates, as its performance as a viral clearance ligand was unacceptable.

The trimers WRW, KRK, RAA, and KYY all exhibited breakthrough of PPV in the presence of plasma proteins after the first three column volumes (Figure 2). There was no detectable cooperative binding observed for these resins in the flowthrough fractions tested. Of these resins, only WRW was able to completely clear all detectable PPV in the first three column volumes from 7.5% human blood plasma. With optimization of the peptide density and spacer length, this resin

may be able to clear PPV in all nine column volumes of challenge solution containing 7.5% human blood plasma. Human blood plasma also contains many different proteins, and only one or two may be interfering with the binding of PPV. For example, if albumin is the predominant protein binding to the resin, then the peptide may be able to clear PPV very well from a solution that contains other proteins but not albumin. In this case, WRW has the potential to be used effectively for final purification of a pure protein with excellent removal efficiency.

Chromatographic beads are not the most efficient way to remove large particles from process streams. The viruses, having a diameter on the same order of magnitude as the pore diameter, have small diffusion coefficients in the pores of the beads, and viruses quickly clog the pores. Consequently, the accessible surface area of the beads is mainly associated with the outside surface of the bead, and the inner pore surface is not available for binding. Membranes have a better geometry for binding of particles such as viruses, as there are not any diffusional limitations. However, the screening of a combinatorial library of peptides is difficult to do on a membrane surface. The SPOT method, developed by Ronald Frank (41), is used to produce peptide libraries on a cellulose membrane surface, but if done manually, only several hundreds of peptides can be created in 2–3 days (42). This is a small library compared to the thousands of peptides that can be screened on chromatographic beads. In addition, the binding to a peptide on cellulose fibers may be quite different from that observed on other membrane materials. There are currently no large ligand libraries on any membrane surface that is likely to be used for large-scale virus removal. This study provides proof-of-concept that peptides have the ability to remove viruses specifically. In the future, it may be beneficial to change the geometry of the support for improved access of all of the ligands to the viral particle, but currently the bead geometry offers a better screening platform.

#### 4. Conclusions

Small trimeric ligands that specifically bind to porcine parvovirus were isolated from a solid-phase peptide library. In PBS, 100% of detectable infectious virus was removed from solution for every fraction that was tested, up to nine column volumes. This demonstrates the potential of these peptides for use in virus removal from samples of relatively simple composition, such as for water purification applications. In more complex mixtures, such as 7.5% human blood plasma, peptide WRW was able to remove all detectable infectious viruses in the first three column volumes. This is impressive for a ligand that contains only three amino acids, as most peptide ligands are a minimum of six amino acids in length. Enhanced specificity and binding affinity may be found using an increased number of amino acids in the ligand, and this is currently being examined. Tethering one or more of the ligands to a membrane with more suitable geometry may improve virus removal efficiency from complex mixtures. The ligands could also be optimized for application to specific process streams, so that a single ligand must only compete with one therapeutic protein, thereby overcoming competitive binding and facilitating use as an efficient virus absorbent.

#### Acknowledgment

The authors would like to thank Pathogen Removal and Diagnostic Technologies (PRDT), a joint venture between the American Red Cross and ProMetic BioSciences Inc., and the NIH/NCSU Molecular Biotechnology Training Program for

funding. The authors also thank Drs. Dennis T. Brown and Raquel Hernandez for their training in cell culture techniques.

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Received October 30, 2007. Accepted April 7, 2008.

BP070412C





医薬品 研究報告 調査報告書

識別番号・報告回数		報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
一般的名称	-	研究報告の 公表状況	http://www.fda.gov/cber/gdlns/natparo.pdf	公表国	米国
販売名(企業名)	-				
研究報告の概要	<p>パルボウイルス B19 DNA を検出するための実施手順に関するガイダンス案が示された。</p> <ul style="list-style-type: none"> <li>全ての血漿由来製剤について、製造プール中のパルボウイルス B19 DNA のウイルス負荷を確実に 10<sup>4</sup>IU/mL 以下とするため、製造過程の品質管理検査としてパルボウイルス B19 の NAT を実施すべきである。</li> <li>ミニプールサンプルのパルボウイルス B19 の NAT 検査は 10<sup>6</sup>IU/mL 以上の個別ユニットを検出できる感度とすべきである。</li> <li>個別ユニットのパルボウイルス B19 DNA の力価が 10<sup>6</sup>IU/mL 以上であることがわかった場合、又は、製造用プール血漿で 10<sup>4</sup>IU/mL のパルボウイルス B19 DNA を上回る可能性がある場合、その後の製造に使用すべきではない。</li> </ul> <p>原料血漿及び回収血漿において、パルボウイルス B19 DNA を検出するため、ならびに製造プールにおけるパルボウイルス B19 DNA のウイルス負荷が 10<sup>4</sup>IU/mL 以下であることを示すために用いるパルボウイルス B19 の NAT 検査の精度、感度、特異度、再現性及びその他の性能特性を示すバリデーションデータを維持管理すべきである。</p>				<p>使用上の注意記載状況・ その他参考事項等</p> <p>慎重投与(次の患者には慎重に投与すること)</p> <ul style="list-style-type: none"> <li>溶血性・失血性貧血の患者 [ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。]</li> <li>免疫不全患者・免疫抑制状態の患者 [ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、持続性の貧血を起こすことがある。]</li> </ul> <p>重要な基本的注意</p> <p>(1) 本剤の原材料となる… [スクリーニング項目、不活化・除去工程]・投与に際しては、次の点に十分注意すること。</p> <p>1) 血漿分画製剤の現在の製造工程では、ヒトパルボウイルス B19 等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。</p> <p>妊婦、産婦、授乳婦等への投与 妊婦又は妊娠している可能性のある婦人には治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。 [妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルス B19 の感染の可能性を否定できない。感染した場合には胎児への障害 (流産、胎児水腫、胎児死亡) が起こる可能性がある。]</p>
	<p>報告企業の意見</p> <p>血漿由来製剤製造用原料血漿に関するパルボウイルス B19 の NAT 検査に関するガイダンス案の情報である。 当社血漿分画製剤は最終製品において NAT 検査を行い、パルボウイルス B19 DNA 陰性であることを確認している。</p>	<p>今後の対応</p> <p>今後ともパルボウイルス B19 に関する血漿分画製剤の安全性に関する情報に留意していく。</p>			

# Guidance for Industry

## Nucleic Acid Testing (NAT) to Reduce the Possible Risk of Parvovirus B19 Transmission by Plasma-Derived Products

### DRAFT GUIDANCE

**This guidance document is for comment purposes only.**

Submit comments on this draft guidance by the date provided in the *Federal Register* notice announcing the availability of the draft guidance. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. Submit electronic comments to either <http://www.fda.gov/dockets/ecomments> or <http://www.regulations.gov>. You should identify all comments with the docket number listed in the notice of availability that publishes in the *Federal Register*.

Additional copies of this draft guidance are available from the Office of Communication, Training and Manufacturers Assistance (HFM-40), 1401 Rockville Pike, Suite 200N, Rockville, MD 20852-1448, or by calling 1-800-835-4709 or 301-827-1800, or from the Internet at <http://www.fda.gov/cber/guidelines.htm>.

For questions on the content of this guidance, contact Mahmood Farshid, Ph.D., at 301-496-0952, or by Fax at 301-402-2780.

U.S. Department of Health and Human Services  
Food and Drug Administration  
Center for Biologics Evaluation and Research  
July 2008

**Contains Nonbinding Recommendations**

*Draft – Not for Implementation*

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**Guidance for Industry**

**Nucleic Acid Testing (NAT) to Reduce the Possible Risk of  
Parvovirus B19 Transmission by Plasma-Derived Products**

*This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.*

**I. INTRODUCTION**

We, FDA, are issuing this guidance to provide you, manufacturers of plasma-derived products, with recommendations for performing parvovirus B19 nucleic acid testing (NAT) as an in-process test for Source Plasma and recovered plasma used in the further manufacturing of plasma-derived products. Such testing will identify and help to prevent the use of plasma units containing high levels of parvovirus B19. This guidance also recommends how to report to the FDA implementation of parvovirus B19 NAT.

We recognize that in the current business practice for parvovirus B19 NAT in-process testing, several weeks can elapse between collection of the units of Source Plasma or recovered plasma and identification of B19 NAT-positive pools or units. We encourage manufacturers of plasma-derived products to employ practices that will reduce the time between product collection and in-process testing to allow for the meaningful notification of blood and plasma collection establishments of positive test results within the dating period of components.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

**II. BACKGROUND**

Parvovirus B19 is a small, non-enveloped single strand DNA virus. This virus is highly resistant to all commonly used inactivation methods, including heat and solvent/detergent (S/D) treatment, and is also difficult to remove because of its small size. The parvovirus B19 can be

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transmitted by blood components and certain plasma derivatives, and may cause morbidity to susceptible recipients such as pregnant women (and their fetuses exposed in utero), persons with underlying hemolytic disorders, and immune compromised individuals (Refs. 1 and 2). The disease transmission by transfusion of blood components is rare; however, extremely high levels of parvovirus B19, up to  $10^{12}$  IU/mL, in plasma of acutely infected but asymptomatic donors may present a greater risk in plasma derivatives due to pooling of large numbers of plasma units in the manufacture of these products. The virus can be detected by NAT in plasma pools when there are high levels of parvovirus B19 DNA in viremic donations. For example, the parvovirus B19 DNA can be detected in various plasma-derived products, particularly in coagulation factors (Refs. 3 and 4). There have been a few reports of parvovirus B19 infection associated with the administration of coagulation factors (Refs. 5 and 6) and S/D Treated Pooled Plasma (Refs. 1 and 7). Parvovirus B19 DNA is less frequently detected in albumin and immunoglobulin products and, when detected, the levels are usually low. There are no confirmed reports that albumin and immunoglobulin products have transmitted parvovirus B19 infection.

We have held or participated in several meetings to discuss the potential risk of parvovirus B19 infection by plasma-derived products, and the strategy for reducing such risk. The meetings included FDA-sponsored NAT workshops in 1999 and 2001 (Refs. 8 and 9), Blood Products Advisory Committee (BPAC) meetings in 1999, and 2002 (Refs. 10, 11, and 12), the National Heart, Lung, and Blood Institute-sponsored Parvovirus B19 workshop in 1999 (Ref. 1), and an ad hoc Public Health Service (PHS) panel in 2002 (discussed at the 2002 BPAC meeting (Ref. 12)). In these meetings, it was recognized that the scientific data indicate that parvovirus B19 is highly resistant to the available viral inactivation methodologies, and is difficult to remove because of its small size. The viral inactivation/removal steps routinely used in the manufacturing process of plasma-derived products do not alone appear to be sufficient to completely clear the virus if high viral load is present in the starting material. Therefore, in these meetings, a common recommendation for mitigating the risk of parvovirus B19 transmission by plasma derivatives has been to limit the virus load in the manufacturing plasma pool by testing the plasma donations for high titer parvovirus B19 DNA, using a minipool format. This viral load reduction strategy combined with the ability of the manufacturing process to clear the residual virus could greatly reduce the risk of parvovirus B19 infection by plasma-derived products.

The recommended limit in this guidance for viral load of parvovirus B19 DNA in the manufacturing plasma pool (i.e., not to exceed  $10^4$  IU/mL) was primarily derived from studies that were conducted on the transmission of parvovirus B19 associated with S/D Treated Pooled Plasma (Refs. 1, 7, and 10). In principle, testing in a minipool format to measure the viral load for parvovirus B19 DNA in a manufacturing plasma pool is acceptable in order to exclude only the high-titer plasma donations, thereby avoiding too great a loss of plasma for further manufacturing. Furthermore, during the viremic period for parvovirus B19 infected donors, which can be very lengthy, low levels of parvovirus B19 coexist with parvovirus B19 antibodies (potentially complexing with and neutralizing the virus). Therefore, it is undesirable to remove plasma units with low levels of B19 DNA, because it would diminish the parvovirus B19 antibody levels in plasma pools and in some of the resulting plasma-derived products (Refs. 13 and 14).

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### III. RECOMMENDATIONS

We recommend that you implement the following procedures to detect the presence of parvovirus B19 DNA:

- For all plasma-derived products, you should perform parvovirus B19 NAT as an in-process quality control test to ensure that the viral load of parvovirus B19 DNA in the manufacturing pools does not exceed  $10^4$  IU/mL.
- Use parvovirus B19 NAT on minipool samples to screen plasma units intended for further manufacturing into plasma-derived products. The sensitivity of the NAT assay, in any size minipool, should be at least  $10^6$  IU/mL for detection of any single donation when tested in the minipool (i.e., if the titer of an individual unit is  $10^6$  IU/mL or higher, the test result on the minipool will be positive). Primers and probes selected for parvovirus B19 NAT should detect all known genotypes of the virus (Ref. 15).
- When identified, you should not use individual plasma units intended for further manufacturing into plasma-derived products, when such units are found to have a titer of parvovirus B19 DNA at or above  $10^6$  IU/mL, or when use of a positive unit might result in plasma manufacturing pools exceeding a parvovirus B19 DNA titer of  $10^4$  IU/mL.

You should maintain validation data demonstrating the accuracy, sensitivity, specificity, reproducibility, and other performance characteristics of the parvovirus B19 NAT assay used for the detection of parvovirus B19 DNA in the Source Plasma and recovered plasma, and for demonstrating that the viral load of parvovirus B19 DNA in the manufacturing pool does not exceed  $10^4$  IU/mL.

If the recommendations are implemented, you must notify FDA of the changes to an approved application under 21 CFR 601.12(c)(5) ("Supplement-Changes Being Effected"), and submit the information required in 21 CFR 601.12(b)(3)(i) through (vii).

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