Inactivation and removal of Zika virus during manufacture of plasma-derived medicinal products

Johannes Blümel, Didier Musso, Sebastian Teitz, Tomoyuki Miyabayashi, Klaus Boller, Barbara S. Schnierle, and Sally A. Baylis

BACKGROUND: Zika virus (ZIKV) is an emerging mosquito-borne Flavivirus of major public health concern. The potential for ZIKV transmission by blood transfusion has been demonstrated; however, inactivation or removal of ZIKV during the manufacture of plasma-derived medicinal products has not been specifically investigated.

STUDY DESIGN AND METHODS: Inactivation of ZIKV by pasteurization and solvent/detergent (S/D) treatment was investigated by spiking high-titer ZIKV stocks into human serum albumin and applying either heat or adding different mixtures of S/D reagents and assaying for infectious virus particles. Removal of ZIKV was evaluated using filters of differing pore sizes (75, 40, 35, and 19 nm), assaying for infectious virus and RNA. Electron microscopy was performed to determine the size of ZIKV particles. Neutralization of virus infectivity by immunoglobulins was investigated.

RESULTS: ZIKV was effectively and rapidly inactivated by liquid heat treatment as well as by various mixtures of S/D reagents with reduction factors more than 4 log, in each case. Effective reduction of ZIKV infectivity was demonstrated for virus filtration for filters with average pore sizes of not more than 40 nm, although a significant proportion of virus RNA was detected in the 40- to 35-nm filtrates likely due to the presence of subviral particles observed by electron microscopy. None of the immunoglobulin preparations investigated neutralized ZIKV infectivity.

CONCLUSIONS: Pasteurization and S/D treatment very rapidly inactivated ZIKV and filters with a pore size of not more than 40 nm removed all infectious ZIKV, demonstrating the effectiveness of these virus reduction strategies used during the manufacture of plasma-derived medicinal products.

Zika virus (ZIKV) was first isolated in 1947 from the blood of a febrile sentinel rhesus monkey in the Zika forest in Uganda, Africa, and from mosquitoes in 1948 in the same area. The first human infection was reported in Nigeria in 1954. Over the following 60 years, fewer than 20 human infections have been reported in Asia and Africa. In 2007, a first outbreak of ZIKV was reported on Yap Island, Federated States of Micronesia in the Pacific; a second one occurred in French Polynesia, in the Pacific, in 2013 or 2014 with the description of the first severe complications of ZIKV infections. Subsequently, ZIKV has spread through other Pacific islands. In 2015, ZIKV emerged in Brazil and subsequently spread in South America and the Caribbean. From 2007, ZIKV circulation has been reported in 64 countries and territories and the first ZIKV circulation has been detected in 42 of them.

Infection with ZIKV is usually asymptomatic or results in mild disease typically presenting with fever, headache, malaise, conjunctivitis, and macropapular skin rash. Severe complications related to ZIKV have been

ABBREVIATIONS: BVDV = bovine viral diarrhea virus; TCID50 = tissue culture infectious dose 50; TNBP = tri-n-butyl phosphate; WNV = West Nile virus; ZIKV = Zika virus.

From the Paul-Ehrlich-Institut, Langen, Germany; Institut Louis Malardé, Tahiti, French Polynesia; and Asahi Kasei, Cologne, Germany.

Address reprint requests to: Johannes Blümel, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, 63225 Langen, Germany; email: johannes.bluemel@pei.de.

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described in adults, principally Guillain-Barré syndrome, and infants, principally microcephaly in the fetus and neonates. Recent studies demonstrated the neurotropism of ZIKV.

ZIKV is a member of the Flaviviridae and is mainly transmitted by the bite of infected female mosquitoes of the Aedes species including the widespread Ae. aegypti and Ae. albopictus. Other routes of transmission of ZIKV including sexual contact, mother-to-child transmission, and blood transfusion have been reported. Since the detection of asymptomatic blood donors viremic for ZIKV in French Polynesia, and the report of suspected cases of ZIKV transmission by blood transfusion in Brazil, ZIKV is considered as a new challenge for blood transfusion. The Food and Drug Administration and the World Health Organization have issued recommendations to prevent ZIKV transmission by blood transfusion. Therefore, there are concerns about safety of blood components and products for transfusion as well as the safety of plasma-derived medicinal products given the pathogenic potential of ZIKV. It has been recently demonstrated that amotosalen and UVA light illumination leads to significant inactivation of ZIKV and pathogen inactivation is an option for reduction of ZIKV in blood components. In this study, we have investigated the inactivation and removal of ZIKV by procedures used during the manufacture of plasma-derived medicinal products.

MATERIALS AND METHODS

Viruses and cells

African green monkey kidney (Vero) E6 cells (ATCC CRL-1586) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and antibiotics. The ZIKV strain PF13/251013-18 was isolated in 2013 from the serum of a French Polynesian patient. Virus was propagated in Vero E6 cells using DMEM supplemented with 2% FBS. Virus-containing supernatant was harvested 8 to 10 days postinfection, clarified by low-speed centrifugation, and stored at -80°C.

Virus titration

Infectious ZIKV was quantified by endpoint titration on Vero E6 cells determining the tissue culture infectious dose 50 (TCID50, i.e., the dose where 50% of cell cultures are infected). Briefly, Vero E6 cells were grown to confluence in 96-well microtiter plates and inoculated with a 50 μL sample from a dilution series of the respective test item. The samples were then incubated in 5% CO2 at 37°C for 7 days and examined for cytopathic effect. Infectivity titers from end point titration were calculated by maximum likelihood algorithm using computer software (ClickIT, provided by Baxter). If no virus was detected, the detection limit was calculated by Poisson distribution. Two to six 96-well microtiter plates were inoculated with test item to increase sensitivity of the assay (large-volume plating).

Heat inactivation in cell culture medium and human serum albumin

Medium (DMEM without serum) was preheated to 56.0°C in a water bath. The temperature was measured throughout the experiment by monitoring an unspiked medium sample incubated in parallel using a calibrated thermometer. Virus was prefiltered using a 0.45 μM filter, to remove viral aggregates. Twenty milliliters of preheated DMEM was spiked at a ratio of 1:20 with ZIKV. The study was performed in duplicate. Samples were withdrawn after 10 minutes, 30 minutes, 1 hour, and 2 hours of heat-treatment. Samples were immediately diluted 1:10 in cold cell culture medium and titrated on Vero E6 cells.

Commercial 5% and 25% human serum albumin (HSA) was heated to 58.0°C in water bath. The temperature was measured throughout the experiment by monitoring an unspiked albumin sample incubated in parallel using a calibrated thermometer. Twenty milliliters of preheated albumin was spiked with ZIKV at a ratio of 1:20. Samples were withdrawn after 10 minutes, 30 minutes, 1 hour, and 2 hours of heat treatment. Samples were immediately diluted 1:10 in cold cell culture medium and titrated on Vero E6 cells.

Solvent/detergent treatment

Inactivation was studied using two mixtures of solvent/detergent (S/D) reagents, that is, 1% Tween 80 and 0.3% tri-n-butyl phosphate (TNBP) or 0.2% sodium deoxycholate and 0.3% TNBP respectively. HSA (5%) was mixed with S/D reagents and brought to 27°C in a water bath for treatment with Tween/TNBP or 29°C for treatment with sodium deoxycholate/TNBP. ZIKV was added at a ratio of 1:11 ratio and samples were taken after 5 minutes, 15 minutes, 30 minutes, 1 hour, and 2 hours. The study was performed with a final ZIKV concentration of 6.4 ± 0.3 log TCID50/mL, before processing. Samples were immediately diluted 1:100 in cell culture medium and inoculated onto indicator cells. Samples without S/D reagents were spiked with ZIKV as a control for the infectious virus titer in the spiked matrix after 0 minutes and 2 hours.

Virus filtration

A 0.5% HSA solution was spiked with ZIKV and processed through 0.1-μm syringe filters (Sartorius). The prefiltered solution was processed through a 0.001 μm2 35N or 20N virus filter (Planova, Asahi Kasei). Filter loads were 40 L/m2. In another experiment, 100 mL of ZIKV-spiked albumin was processed through a series of Planova filters with different average pore diameters, that is, 75 nm (Planova
RESULTS

INACTIVATION OF ZIKA VIRUS

Electron microscopy
For plastic embedding, Vero cells were infected with ZIKV at a multiplicity of infection of approximately 0.1. Eight days postinfection, the cells were fixed using 2.5% glutaraldehyde in Dulbecco’s phosphate-buffered saline for 45 minutes and embedded in Epon resin as previously described.27 Ultrathin sections were cut and stained with 2% uranyl acetate followed by 2% lead citrate. Sections were examined in an electron microscope (Model EM902, Zeiss).

Neutralization assay
Neutralization reactions (final volume, 500 μL) were set up by adding 5000 TCID$_{50}$ of ZIKV to serial 1:5 dilutions of commercial normal immunoglobulin preparations. The immunoglobulins varied in concentration, that is, 5% (50 mg/mL), 10% (100 mg/mL), and 16% (160 mg/mL). After being incubated for 2 hours at 37°C, the material was inoculated onto Vero E6 cells (eight replicas per dilution step).

Heat inactivation of ZIKV
Inactivation of ZIKV in preheated cell culture medium was investigated first. ZIKV was inactivated after 5 minutes at 56°C (Table 1) in the absence of serum. Titration of non–heat-treated control samples verified input concentration of ZIKV at 5.07 and 5.67 log TCID$_{50}$/mL. The detection limit of the assay was not more than 0.33 TCID$_{50}$/mL resulting in log reduction factors of at least 4.74 and at least 5.34, respectively. Liquid heat treatment of plasma proteins is usually performed at 60°C for 10 hours (pasteurization). For HSA, pasteurization is performed directly on the product in the final container. In our experiments, the temperature for inactivation was set to 58°C, that is, 2°C below the standard manufacturing conditions for treatment of albumin to evaluate the robustness of the procedure. Titration of nonheated albumin samples verified input concentrations of 5.43 ± 0.31 and 5.47 ± 0.31 log TCID$_{50}$/mL. The kinetics of ZIKV inactivation in commercial samples of 5 and 25% HSA are shown in Fig. 1. Rapid reaction kinetics were observed and by 10 minutes, the first time point evaluated, no infectious virus was detected. Logarithmic reduction values of at least 5.09 and at least 5.13 could be demonstrated for the 60-minute samples using large-volume plating assay with a 95% detection limit of 0.34 log TCID$_{50}$/mL.
Inactivation kinetics by S/D treatment

We studied ZIKV inactivation by two commonly used mixtures of S/D reagents, that is, final concentrations of 1% Tween 80 and 0.3% TNBP or 0.2% sodium deoxycholate and 0.3% TNBP, using 0.5% albumin as a model protein matrix. ZIKV was inactivated below the detection limit after 15 minutes (Fig. 2) in both mixtures of S/D reagents.

Titration of spiked samples without S/D reagents verified input concentrations of $5.55 \pm 0.31$ and $5.54 \pm 0.31$ log TCID$_{50}$/mL. Logarithmic reduction values of at least 4.21 and at least 4.20 could be demonstrated for the 120-minute samples using large-volume plating assay with a 95% detection limit of 1.34 log TCID$_{50}$/mL.

Virus removal by filtration

Inoculation of ZIKV-spiked test material on Vero E6 cells indicated the presence of infectious particles. Electron micrographs of ZIKV-infected cells showed virus particles (approx. 48-50 nm) being released from cells (Figs. 3A and 3B) as well as the intimate association of virus particles with the endoplasmic reticulum (Fig. 3C) and the development of vesicles and virus factories in infected cells (Fig. 3E).

Smaller particles of approximately 35 nm were also observed being released from cells and these may represent defective particles (Fig. 3D). RNA quantification after nuclease treatment indicated the virus particle-associated (encapsidated) RNA or virus particle concentration. We used carefully manufactured Planova filters with defined average pore diameters, to characterize removal of infectious and noninfectious virus particles from cell culture supernatants. Serial filtration of virus preparations through the filters with defined pore diameters of 75, 40, 35, and 19 nm showed that infectious virus particles were completely removed by the 40-nm filters (i.e., log reduction values of more than 6.72) while significant fraction of particle-associated RNA penetrated the 40- and 35-nm filters (Table 2, Samples 1–5). Electron microscopy revealed that most particles observed in cells and those being released were larger (approx. 48-50 nm) than the pore size of virus filters removing effectively infectious virus. Occasionally smaller particles were observed of approximately 35 nm, and these may represent the particles associated with the 40- and 35-nm filtrates. Comparing the infectious virus titers with the ZIKV RNA loads, these are in the ratio of approximately 1:100 to 1:1000 suggesting the presence of defective virus particles.

A low concentration of residual virus RNA was found in the 19 nm filtrates (Table 2). This could represent incompletely digested free RNA or RNA associated with proteins or incomplete particles. However, filtration using Planova 35N (Table 2, Runs 6–8) or Planova 20N filters (Table 2, Runs 9–11) confirmed that infectivity can be completely removed with log reduction factors of 6.67 and 6.74 log TCID$_{50}$/mL, respectively.
Virus neutralization

Seven immunoglobulin preparations from a total of five different manufacturers, sourced from North America or Europe, containing 5, 10, or 16% IgG were analyzed. All products failed to demonstrate any evidence of ZIKV neutralization at immunoglobulin dilutions of 1:10 or higher. Nondiluted IgG preparations were not investigated because of their cytotoxic effect.

Controls performed with blood donor plasma sourced from Germany in 2013 were negative. A positive control serum from a patient with recent ZIKV infection showed neutralization up to dilutions of 1:1250 (1:250-1:6250).

**DISCUSSION**

With the spread of ZIKV in Central and South America and predictions about its wider dissemination, concern has been raised about the safety of plasma-derived medicinal products. Soon after ZIKV was first isolated in the 1940s, studies were performed investigating virus stability using homogenates of ZIKV-infected mouse brains. The brain homogenates (10% suspensions), subjected to heat treatment, were inoculated into mice to determine the effectiveness of the heat treatments. The results of the mouse studies showed that heat treatment either at 58°C for 30 minutes or at 60°C for 15 minutes resulted in survival of the animals inoculated with the heat-inactivated virus. These observations fit well with the heat inactivation of ZIKV by pasteurization at 58°C in HSA (5 or 20%) observed in our study with very rapid inactivation of virus even after 5 minutes of heat treatment. Virus spiked into cell culture medium without serum was inactivated in less than 5 minutes after spiking virus into medium preheated to 56°C. One recent study had suggested that ZIKV was more stable than the closely related dengue virus although this study only investigated virus inactivation at 40°C where some instability of dengue virus (Serotypes 2 and 4) was observed, while ZIKV was unaffected. One previous study examined the inactivation of dengue virus by pasteurization at 58°C in HSA (5 or 20%) observed in our study with very rapid inactivation of virus even after 5 minutes of heat treatment. Virus spiked into cell culture medium without serum was inactivated in less than 5 minutes after spiking virus into medium preheated to 56°C. One recent study had suggested that ZIKV was more stable than the closely related dengue virus although this study only investigated virus inactivation at 40°C where some instability of dengue virus (Serotypes 2 and 4) was observed, while ZIKV was unaffected. One previous study examined the inactivation of dengue virus by pasteurization of albumin and, similar to ZIKV in our studies, was rapidly inactivated at 59°C after 5 minutes. A recent report of the heat sensitivity of different alphaviruses, using a heat treatment protocol of 56°C for 30 minutes for inactivation of serum samples for virus neutralization testing, demonstrated that not all viruses were inactivated. The alphaviruses have some similarities with flaviviruses such as ZIKA (both are arboviruses), and the study by Park and colleagues shows that there may be differences in the susceptibility of different viruses to inactivation methods and specific virus studies may be of value. Our studies on West Nile virus (WNV) and with bovine viral diarrhea virus (BVDV), a model animal flavivirus, showed slower reduction of ZIKV using a range of widely used inactivation and removal procedures employed during the manufacture of this class of medicinal products.

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**Fig. 3. Analysis of ZIKV in infected Vero E6 cells. ZIKV factories in infected Vero E6 cells and release of virus particles 8 days postinfection. (A, B, and D) ZIKV particles being released from the plasma membrane. (C) ZIKV particles developing in close association with the endoplasmic reticulum. (E) Membrane vesicles and developing ZIKV particles in the cytoplasm. The scale bars are 200 nm; the scale in panels A-D is the same.**

**TABLE 2. Removal of ZIKV by virus filtration**

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Description</th>
<th>ZIKV RNA copies/mL*</th>
<th>Infectious ZIKV (Log TCID50/mL)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Spiked albumin</td>
<td>9.09</td>
<td>6.36</td>
</tr>
<tr>
<td>2</td>
<td>75-nm filtrate</td>
<td>6.79</td>
<td>4.54</td>
</tr>
<tr>
<td>3</td>
<td>40-nm filtrate</td>
<td>4.87</td>
<td>≤ −0.36</td>
</tr>
<tr>
<td>4</td>
<td>35-nm filtrate</td>
<td>4.72</td>
<td>≤ −0.36</td>
</tr>
<tr>
<td>5</td>
<td>19-nm filtrate</td>
<td>3.36</td>
<td>≤ −0.36</td>
</tr>
<tr>
<td>Run 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Spiked albumin</td>
<td>8.35</td>
<td>6.31</td>
</tr>
<tr>
<td>7</td>
<td>0.1-μm filtrate</td>
<td>8.09</td>
<td>6.31</td>
</tr>
<tr>
<td>8</td>
<td>35-nm filtrate</td>
<td>4.75</td>
<td>≤ −0.36</td>
</tr>
<tr>
<td>Run 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Spiked albumin</td>
<td>8.03</td>
<td>6.38</td>
</tr>
<tr>
<td>10</td>
<td>0.1-μm filtrate</td>
<td>8.10</td>
<td>6.31</td>
</tr>
<tr>
<td>11</td>
<td>19-nm filtrate</td>
<td>2.72</td>
<td>≤ −0.36</td>
</tr>
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</table>

*Log RNA copies/mL after treatment of samples with nuclease. †Log TCID50/mL on Vero E6 indicator cells.
inactivation kinetics when viruses were subject to pasteurization in immunoglobulin solution stabilized with sucrose and matrix may have effect on stability.\textsuperscript{73}

S/D treatment is widely used for inactivation of enveloped viruses in plasma or during the manufacture of plasma-derived medicinal products. Effectiveness of S/D treatment against a broad variety of virus strains from different virus families has been demonstrated.\textsuperscript{34} Treatment of ZIKV with S/D reagents, irrespective of composition, very rapidly inactivated the virus. These rapid inactivation kinetics of ZIKV is similar to our previous observations with BVDV.\textsuperscript{35} Although S/D treatment has been investigated for dengue virus,\textsuperscript{31} studies have been performed using Triton X-100, which is more effective for inactivation of enveloped viruses\textsuperscript{34} and the data are therefore not directly comparable. Inactivation of chikungunya virus (CHIKV) using S/D reagents or heat treatment has also been confirmed.\textsuperscript{36} As concluded in this study, robust inactivation from widely used model viruses such as BVDV can predict inactivation of related emerging viruses such as WNV, chikungunya virus, or ZIKV by treatment with S/D reagents or heat.

Manufacture of many plasma derivatives frequently includes specific virus filters for removal of small viruses such as parvoviruses. While there is little doubt that these filters will remove ZIKV, such filters cannot be applied to complexes of coagulation factors such as Factor VIII associated with von Willebrand factor and filters with large pore sizes (i.e., in the range between 30 and 50 nm) have to be used. Infectious ZIKV particles were retained by 40- and 35- as well as 19-nm virus filters; these data demonstrate that the size of the infectious ZIKV particles exceeds 40 nm and are in line with expectations of the virus particle size of approximately 48 to 50 nm shown developing in and being released from infected Vero E6 cells by electron microscopy as well as recently determined by cryo-electron microscopy.\textsuperscript{30,37} Noninfectious, subviral particles were evidenced by the presence of virus RNA in the filtrates (produced after filtration using the 40- and 35-nm filters). We did not investigate whether virus particles passing the 35-nm filters contained truncated viral genomes. In similar nanofiltration studies using dengue virus, there is a reasonably high virus RNA load in 35-nm filtrates; again this is not associated with infectious virus.\textsuperscript{31} Such observations demonstrate the importance of evaluating infectivity when performing virus filtration studies and not just monitoring distribution of virus RNA in the respective fractions. Therefore, even for virus filters with a pore diameter of 35 nm, effective reduction of ZIKV would be expected. It is likely that the very low ZIKV RNA loads observed in the 19-nm filtrates might be due to incompletely digested virus RNA or RNA protected (in part) by viral proteins. The RT-PCR assay used amplifies small fragment of the virus genome (approx. 86 bp), which would be more easily protected than a larger fragment of several hundred bases.

The lack of ZIKV-neutralizing antibodies in therapeutic immunoglobulins prepared in Europe and North America is unsurprising. This demonstrates lack of exposure of the donor populations to ZIKV and is similar to the situation with WNV early in the North American outbreak before the virus spread widely.\textsuperscript{33}

In summary, ZIKV is sensitive to commonly used inactivation and removal procedures widely used during the manufacture of plasma-derived medicinal products. By analogy to other flaviviruses such as hepatitis C virus (HCV), risk assessment evaluating the safety margins of plasma-derived medicinal products are performed for currently marketed plasma-derived medicinal products, and established clearance for HCV would also be applicable for ZIKV.

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CONFLICT OF INTEREST

ST and TM are employees of Asahi Kasei Corporation. The remaining authors have disclosed no conflicts of interest.

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