BACKGROUND: The ongoing Zika virus (ZIKV) outbreak in the Americas has also raised concerns around the potential for ZIKV transmission via blood products. Plasma-derived products are considered safe, because effective viral-inactivation and removal methods are implemented in their manufacturing processes. However, a recent study has indicated that ZIKV is “thermally stable” compared with the closely related Dengue virus, thus raising the question of whether heat treatments, as embedded in the manufacturing of plasma-derived products, are as effective against ZIKV as was previously shown for other Flaviviruses. Therefore, the sensitivity of ZIKV to heat inactivation was investigated using the pasteurization of human serum albumin (HSA) as an example.

STUDY DESIGN AND METHODS: Heat treatment (58.0 ± 1.0°C for 590 ± 10 minutes) of HSA was investigated for the capacity to reduce ZIKV in two different protein concentrations (5% and 25% HSA). The results were compared with data obtained in identical set-ups for the closely related West Nile virus, tick-borne encephalitis virus, and bovine viral diarrhea virus.

RESULTS: Heat treatment of HSA inactivated ZIKV to below the limit of detection already during the heating phase to 57.0°C, that is, even before the 10-hour incubation at 58.0 ± 1.0°C commenced. For West Nile virus, bovine viral diarrhea virus, and tick-borne encephalitis virus, incubations up to 180 minutes were required to achieve inactivation to below the limit of detection.

CONCLUSION: ZIKV was more sensitive to heat treatment than other members of the Flaviviridae and thus does not pose a concern for plasma products that include a heat treatment in their manufacturing process.

Zika virus (ZIKV), which is currently emerging in the Americas and has potential to extend its range as far north as the southern United States, is primarily transmitted via Aedes mosquito species. However, other routes of transmission, such as perinatal infection and sexual contact, have been reported. The high rate of approximately 80% asymptomatic infections, the report from French Polynesia that 3% of blood donors were positive for ZIKV RNA during an ongoing ZIKV outbreak, and the transfusion transmission of the closely related and similarly transmitted West Nile virus (WNV) and Dengue virus (DENV) suggest a significant risk of ZIKV transmission via blood transfusion. In response to this risk, agencies and blood-collection organizations have adopted policies of donor deferral and started the implementation of nucleic acid testing for ZIKV; however, similar measures have been considered unnecessary for plasma donations because viral inactivation and removal methods are used to clear any potentially present viruses in the manufacturing process for plasma-derived products. The efficacy of these reduction steps has been demonstrated in virus clearance studies using, among others, DENV, WNV, and tick-borne encephalitis virus (TBEV) as well as bovine viral diarrhea virus (BVDV), a frequently used model virus for hepatitis C virus. All of these are members of the family Flaviviridae. From Global Pathogen Safety, Shire (previously Baxalta), Vienna, Austria.

ABBREVIATIONS: BVDV = bovine viral diarrhea virus; DENV = Dengue virus; TBEV = tick-borne encephalitis virus; TCID₅₀ = 50% tissue culture infectious dose; WNV = West Nile Virus; ZIKV = Zika virus.

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of the enveloped, single-stranded RNA \textit{Flaviviridae} family and thus are closely related to ZIKV (\textit{Flavivirus}, \textit{Flaviviridae}). Based on their highly similar structure,\textsuperscript{12} near-identical physicochemical properties can be assumed for all members of the \textit{Flaviviridae}, and the same efficient reduction during the manufacturing processes of plasma-derived products as previously shown for DENV, WNV, TBEV, and BVDV\textsuperscript{8-11} should also apply to ZIKV.

However, in a recent study, a more compact surface of the ZIKV particle and a greater thermal stability when incubated at 40°C were observed compared with DENV.\textsuperscript{13} That report was widely circulated within the transfusion community shortly after its publication\textsuperscript{14} and raised the question whether the heat treatments used during the manufacturing of plasma-derived products are as effective for the inactivation of ZIKV as has previously been shown for other Flaviviruses (Table 1). Therefore, we undertook the current work to verify the sensitivity of ZIKV to heat inactivation using the pasteurization of human serum albumin (HSA) as an example. The results obtained were correlated to previously generated WNV, TBEV, and BVDV inactivation data that were obtained in identical experimental set-ups.\textsuperscript{8,11}

### MATERIALS AND METHODS

#### Cells, viruses, and assays

Vero cells (84113001) used for the propagation and titration of ZIKV were obtained from the European Collection of cell cultures and maintained in TC-Vero medium (Pfizer) supplemented with fetal calf serum (SAFC Biosciences), L-glutamine, gentamycin, nonessential amino acids solution, sodium pyruvate, and sodium carbonate (Life Technologies).

ZIKV strain PRVABC59 was obtained from the Centers for Disease Control and Prevention. This strain was isolated in 2015 from a patient who had returned from Puerto Rico, and the whole genome sequence is available at the National Center for Biotechnology Information (NCBI) (no. KU501215).\textsuperscript{15} ZIKV strain FSS 13025 was obtained from the World Reference Center for Emerging Viruses and Arboviruses (University of Texas Medical Branch, Houston, TX). This strain was isolated in Cambodia in 2010, and the sequence of the polyprotein gene is available at the NCBI (no. JN860885)\textsuperscript{16,17}

Virus infectious titers were determined by using 50% tissue culture infective dose (TCID\textsubscript{50}) assays with serial half-log sample dilutions that were titrated in eightfold replicates in 96-well plates. After incubation for 7 days in humidified CO\textsubscript{2} incubators at 36°C, wells were scored for virus infection by evidence of a cytopathic effect and infectious virus titers (expressed as log\textsubscript{10} TCID\textsubscript{50}/mL) calculated according to the Poisson distribution and the maximum-likelihood method.\textsuperscript{18} The ZIKV stocks used in these experiments had infectivity titers of 6.5 and 5.7 log TCID\textsubscript{50}/mL for the strains PRVABC59 and FSS 13025, respectively.

**Downscaled manufacturing process for HSA**

The reduction study was designed to support the manufacturing process for Shire HSA. The downscaled model, which was established with the target of identity to the manufacturing process, had already been validated in previous studies.\textsuperscript{8,11,19,20} and equivalence to the large-scale process was demonstrated by a comparison of critical process parameters (incubation time and temperature, pH value). Virus reduction factors (RF) (reported in log\textsubscript{10} values) were calculated in accordance with regulatory guidelines.\textsuperscript{21}

HSA is manufactured from human plasma by the modified Cohn-Oncley cold-ethanol fractionation method followed by pasteurization of the final product at 60 ± 1°C for 10 to 11 hours. The capacity to inactivate ZIKV by heat treatment was investigated in conditions least favorable for virus inactivation, that is, at or below the lower limits of incubation temperature and time (58.0 ± 1.0°C for 590 ± 10 minutes), as previously described.\textsuperscript{8,11,19,20} ZIKV stock was used in a 1:20 spike into 5% or 25% HSA (i.e., the highest and lowest possible protein concentrations) obtained from Shire manufacturing facilities in Vienna. Samples were removed and titrated immediately after the virus spike, when the temperature of the spiked material reached 57.0°C and after 15 ± 1 minutes, 30 ± 1 minutes, 60 ± 2 minutes, 120 ± 3 minutes, 360 ± 5 minutes, and 590 ± 10 minutes; and the results were compared with already existing WNV, TBEV, and BVDV inactivation data.\textsuperscript{8,11}

### TABLE 1. Inactivation of DENV, WNV, TBEV, and BVDV by heat treatment during the manufacturing of HSA in minutes until the limit of detection was reached at the latest (log-reduction factor)

<table>
<thead>
<tr>
<th>Virus</th>
<th>3.5% HSA</th>
<th>5% HSA</th>
<th>25% HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV</td>
<td>ND</td>
<td>ND</td>
<td>5 (&gt;4.6)*</td>
</tr>
<tr>
<td>WNV</td>
<td>120 (&gt;8.1)†</td>
<td>120 (&gt;7.4); 180 (&gt;8.3)‡</td>
<td>30 (&gt;6.3; &gt;6.7; &gt;7.9)†‡</td>
</tr>
<tr>
<td>TBEV</td>
<td>60 (&gt;7.0)†</td>
<td>60 (&gt;7.0)†</td>
<td>ND</td>
</tr>
<tr>
<td>BVDV</td>
<td>30 (&gt;6.2)†</td>
<td>30 (&gt;4.1); 120 (&gt;6.4)‡</td>
<td>30 (&gt;6.1; &gt;3.7); 60 (&gt;6.2)†‡</td>
</tr>
</tbody>
</table>

* Data from Xie et al., 2008\textsuperscript{10}; done in 20% HSA instead of 25%.
† Data from Kreil et al., 2003.\textsuperscript{8}
‡ Data from Remington et al., 2004,\textsuperscript{9} and Leydold et al., 2012.\textsuperscript{11}
ND = not done.
RESULTS

Equivalence of the laboratory down-scale heat treatment to the large-scale manufacturing process was demonstrated by comparison of the critical process parameters incubation time, temperature, and pH. All down-scaled heat treatments were done at or below the limits of incubation time and temperature (58.0 ± 1.0°C for 590 ± 10 minutes), representing conditions least favorable for virus inactivation. The spike into HSA did not significantly alter the pH of the matrix (pH of unspiked vs. spiked HSA, 7.02 ± 0.01 vs. 7.00 ± 0.04), as all values were within the product specification of pH 6.7 to 7.3. Heating the spiked HSA up to 57.0°C (time range down-scale, 16-40 minutes vs. approximately 2 hours in manufacturing) already inactivated both strains of ZIKV, the Cambodian strain isolated in 2010, and the recent 2015 isolate from Puerto Rico within the limit of detection (LOD), that is, even before the period of the actual heat treatment (58.0 ± 1.0°C for 590 ± 10 minutes) commenced (Fig. 1a). ZIKV inactivation was not caused by incubation in the matrix (i.e., in 5% or 25% HSA for 590 ± 10 min), because only an insignificant loss in virus infectivity (mean, −0.1 log TCID50/mL; n = 8) was observed in spike controls that were stored at 4°C in parallel to the

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**Fig. 1.** Heat treatment of HSA. Virus-inactivation kinetics are illustrated for incubation at 58.0 ± 1.0°C for 590 ± 10 minutes. Mean log-reduction factors and standard deviations are shown with the number of replicates (n). Arrows (↓) indicate the latest time point at which inactivation to below the limit of detection was achieved in all runs, and asterisks (*) indicate the lower limit of detection toward the end of treatment due to cumulative negative scores. (a) Inactivation of ZIKV in 5% HSA (△; n = 4) and 25% HSA (▽; n = 4); and (b) inactivation of ZIKV, WNV, TBEV, and BVDV are illustrated. Six hundred-minute data plotted for ZIKV actually were measured at 590 minutes, and 30-minute data plotted for TBEV actually were measured at 40 minutes (ZIKV [△; n = 8], WNV [○; n = 4], BVDV [▼; n = 2], TBEV [◇; n = 4]). WNV and BVDV data are from Leydold et al., 201211; TBEV data from Kreil et al., 20038 (done in 3.5% HSA instead of 5%).
process. In duplicate runs, mean RFs of >6.6 and >6.0 log TCID\textsubscript{50} or >5.6 and >5.0 log TCID\textsubscript{50} for ZIKV strain PRVABC59 or ZIKV strain FSS 13025 spiked into 5% and 25% HSA, respectively, were obtained. For comparison, WNV, a virus of significant concern from a blood-product perspective in the United States from the early 2000s, and BVDV, a classical lipid-enveloped model virus per regulatory guidance,\textsuperscript{21} were inactivated somewhat less rapidly. For WNV, no infectious virus was detected at the latest after 180 minutes of incubation (Table 1), and mean RFs of >8.3 log TCID\textsubscript{50} and >7.9 log TCID\textsubscript{50} were obtained in duplicate runs in 5% and 25% HSA, respectively (Fig. 1b).\textsuperscript{8} BVDV was inactivated to below the LOD at the latest after 120 minutes of incubation (Table 1), with RFs of >6.4 log TCID\textsubscript{50} and >6.2 log TCID\textsubscript{50} in 5% and 25% HSA single runs, respectively (Fig. 1b).\textsuperscript{11} For another comparison, inactivation of TBEV, a Flavivirus endemic in Central Europe and North East Asia, was similar, with no infectious virus detected at the latest after 60 minutes of 58°C heat treatment for 600 minutes in 3.5% and 25% HSA, respectively (Table 1 and Fig. 1b).\textsuperscript{8} Contrary to the report of greater thermal stability of ZIKV compared with DENV during an incubation at 40°C,\textsuperscript{13} DENV was the more stable of the two during heat treatment in 25% HSA, because inactivation to below the LOD was observed after 5 minutes for DENV (Table 1), whereas ZIKV already was inactivated during the heating phase to the target temperature (Fig. 1a).

**DISCUSSION**

Possible ZIKV blood transfusion transmissions have already been reported in media announcements from Brazil\textsuperscript{22,23} and in a recent publication,\textsuperscript{24} and the great potential that blood products might contribute to the spread of this virus has resulted in the implementation of measures to avoid donations of ZIKV-positive units,\textsuperscript{7} even the permitted use of a nonlicensed screening assay under an investigational new drug application for areas of active ZIKV transmission, such as Puerto Rico.\textsuperscript{25} Because of the significant virus reduction afforded by the dedicated inactivation and removal steps in the manufacturing processes for plasma-derived products, similar measures have been considered unnecessary for plasma donations used for fractionation.\textsuperscript{6,7} The effectiveness of reduction steps implemented in the manufacturing processes of plasma-derived products has been shown in virus verification studies that used, among others, members of the *Flavivirus* genus, namely, DENV, WNV, BVDV, and TBEV.\textsuperscript{8-11} The current investigation verified that heat treatment of HSA is as effective in the inactivation of ZIKV as has previously been shown for other relevant target and model viruses.\textsuperscript{8-11} Contrary to what might be expected, but similar to results obtained from heat treatment of hepatitis A virus,\textsuperscript{20} inactivation of the Flaviviruses was consistently more rapid in the high-protein matrix of 25% HSA rather than the lower protein matrix of 3.5% and 5% HSA (Table 1). The results confirm, as previously stated,\textsuperscript{11} that BVDV is the most heat-resistant member of the *Flaviviridae* family that has been evaluated and that BVDV is the model virus of choice for hepatitis C virus and all other Flaviviruses in pasteurization studies, which should also include low HSA protein concentrations.

The obtained results clearly show that the reported “thermal stability” of ZIKV versus another Flavivirus at 40°C,\textsuperscript{13} which might be of relevance for the epidemiological transmission of ZIKV, is not of concern for the safety of plasma products. ZIKV was the most rapidly inactivated virus during heat treatments of all the *Flaviviridae* members evaluated, which confirmed that any reservations around the safety of plasma products are truly unwarranted.

**ACKNOWLEDGMENTS**

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**CONFLICT OF INTEREST**

Both authors are employees of Shire. Thomas R. Kreil has stock interest in the company.

**REFERENCES**


