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Short communication

Inactivation of Zika virus in human breast milk by prolonged storage or pasteurization

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ABSTRACT

Zika virus infection during pregnancy poses a serious risk for pregnant women as it can cause severe birth defects. Even though the virus is mainly transmitted via mosquitos, human-to-human transmission has been described. Infectious viral particles have been detected in breast milk of infected women which raised concerns regarding the safety of breastfeeding in areas of Zika virus transmission or in case of a suspected or confirmed Zika virus infection. In this study, we show that Zika virus is effectively inactivated in human breast milk after prolonged storage or upon pasteurization of milk.

Zika virus (ZIKV) infections have been declared to be a Public Health Emergency of International Concern on 1st of February 2016 by the World Health Organization (WHO) (WHO, 2016a). The virus was originally discovered in 1947 in Uganda, however; it received little attention until 2007 and 2013/2014 when a large outbreak was identified in Micronesia and French Polynesia, respectively (Duffy et al., 2009; Cao-Lormeau et al., 2016). Most importantly, during this and later outbreaks in Brazil, neurological complications including an increased incidence of Guillain–Barré syndrome and fetal microcephaly were observed and could be linked to the infection (Cao-Lormeau et al., 2016; Brasil et al., 2016). Since then, the virus was able to spread rapidly and by now 70 countries and territories have reported cases of ZIKV infection (WHO, 2016b). Transmission occurs mainly through mosquitos, with the most important and common vectors for ZIKV being Aedes mosquitoes. However, other modes of transmission, including sexual transmission have been implicated (Lessler et al., 2016; Arsuaga et al., 2016). Infection poses a serious risk especially for pregnant women, as congenital anomalies can occur in the fetus through trans-placental transmission (Schuler-Faccini et al., 2016). Notably, ZIKV RNA has been detected in breast milk of infected mothers and the virus has been found to be infectious in cell culture (Dupont-Rouzeyrol et al., 2016; Besnard et al., 2014). These findings raised questions regarding the safety of breastfeeding in areas of ZIKV transmission. In June 2016, the WHO has published a guideline summarizing the current evidence regarding the risk of ZIKV transmission via breastfeeding in which mothers with suspected, probable or confirmed ZIKV infection are encouraged to breastfeed their children, as the beneficial effects of breastfeeding preponderate any potential risk of ZIKV transmission via breast milk (WHO Geneva, 2016). Here, we aimed to elucidate the stability and inactivation of infectious ZIKV in human breast milk.

To this end, we collected milk samples from three healthy human donors after ethical approval by the ethics commission of Hanover Medical School, Hanover, Germany. All mothers provided written informed consent for the collection of samples and subsequent analysis. Milk was collected freshly and stored at −80 °C

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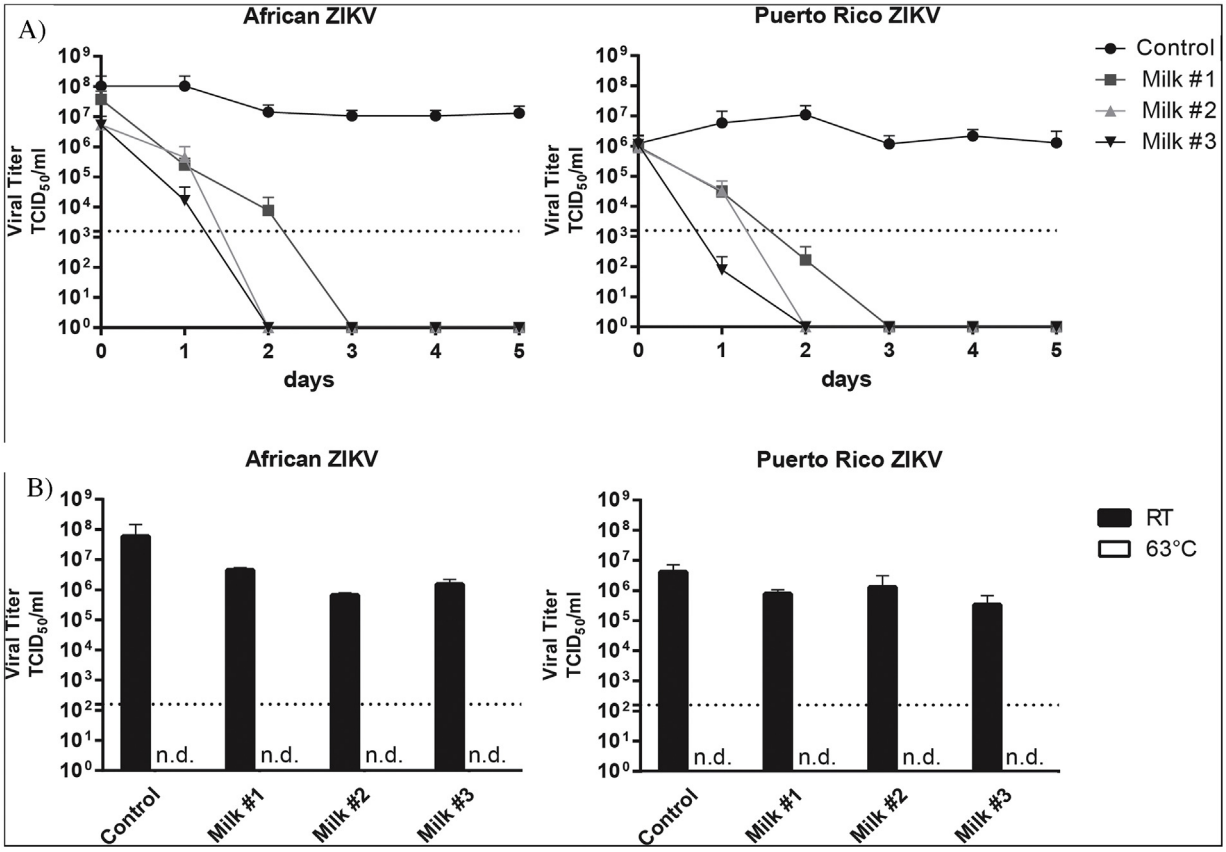

Fig. 1. Inactivation of ZIKV in human breast milk. (A) The African lineage strain of ZIKV and circulating strain from Puerto Rico were mixed at a ratio of 1:10 with human breast milk from three healthy donors (milk #1–3) or as control with cell culture medium. The mixture was used to infect Vero B4 cells in a limiting dilution assay and viral titers were determined as $TCID_{50}$/ml 72 h post infection. Samples were used directly in infection experiments (t = 0) or stored for up to 5 days at 4 °C before titration. Dashed line indicates the detection limit. Depicted are the means + SD from three individual experiments. (B) ZIKV and milk/medium were mixed as described above. Samples were kept at room temperature (RT) or 63 °C for 30 min before viral titers were determined by limiting dilution assay. Dashed line indicates the detection limit. Depicted are the means + SD from three individual experiments, n.d. not detected.

until further use. To analyze viral stability in human breast milk, we incubated the African lineage strain of ZIKV (MP-7501) or the circulating strain from Puerto Rico (PRVABC59) at a ratio of 1:10 (corresponding to 1.11×10^6 $TCID_{50}$/mL) with human breast milk or as control cell culture medium (Dulbecco's modified minimal essential medium (DMEM); Life Technologies, supplemented with 2 mM L-glutamine (Invitrogen), non-essential amino acids (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 100 IU/mL penicillin (Invitrogen), and 10% fetal bovine serum). The low passage African lineage strain of ZIKV (MP-7501) and circulating strain from Puerto Rico (PRVABC59) have been obtained from Culture Collections of Public Health England (UK) and passaged twice on A. albopictus C6/36. We either immediately infected 1×10^4 Vero B4 cells (kindly provided by M. Müller, University of Bonn Medical Centre) with the virus/milk mixture in a limiting dilution assay or stored the mixed samples at 4 °C for several days before virus titration. Virus titer was determined as Tissue Culture Infectious Dose 50 ($TCID_{50}$) 72 h post infection by ZIKV-specific immunostaining. Briefly, cells were washed with PBS, fixed for 10–20 min with 4% PFA, and washed with PBS containing 0.1% saponin. Cells were incubated with primary mouse anti-flavivirus group antigen 4G2 antibody (ATCC HB-112) in 0.3% saponin for 45 min at 37 °C before washing and addition of HRP-conjugated rabbit anti-mouse antibody (Dako) for 45 min at 37 °C. 3-amino-9-ethylcarbazol (AEC) substrate was added for 15 min at RT and the viral titer was determined. As depicted in Fig. 1A, we found a reduction of viral titers in a time-dependent manner starting with one day of storage. After 1–3 days (depending on the donor), virus became inactivated and viral infectivity was no longer detectable (Fig. 1A). No differences regarding viral stability could be observed between the different ZIKV strains. Importantly, virus remained stable for up to 5 days in cell culture medium indicating that human breast milk inactivates ZIKV in a time-dependent manner (Fig. 1A). We further aimed to explore easy and cost-effective measures to rapidly inactivate infectious viral particles in contaminated breast milk samples. Pasteurization of raw milk has been described to constitute an effective method to prevent disease outbreaks (Lucey, 2015). To test the effect of milk pasteurization on ZIKV stability, we mixed virus with milk or as control cell culture medium at a ratio of 1:10 before heating the samples at 63 °C or keeping them at room temperature (RT) for 30 min. Pasteurization of milk or medium reduced ZIKV infectivity below the limit of detection, independent of the milk donor or virus strain (Fig. 1B). These results imply that administration of milk pasteurization prior infant feeding might help to prevent viral transmission in cases of doubt. In conclusion, we demonstrate that ZIKV can be efficiently inactivated in human breast milk. Two case-report studies, analyzing three mother-infant pairs, have provided evidence regarding the presence of ZIKV in breast milk samples of infected women, with one study demonstrating that viral particles are infectious in cell culture (Dupont-Rouzeyrol et al., 2016; Besnard et al., 2014). These findings have raised questions regarding the safety of breastfeeding in the context of a ZIKV infection. Breastfeeding is essential to the development of the infant and has considerable benefits for mother and child. The WHO recom-

mends that women should start breastfeeding within 1 h of birth, irrespective of a suspected, probable or confirmed ZIKV infection (WHO Geneva, 2016). To further evaluate the risk of a potential ZIKV transmission via breastfeeding, we analyzed the stability of ZIKV in breast milk. We demonstrate that human breast milk is able to inactivate ZIKV in a time-dependent manner. It has been described before that human breast milk acts antiviral against several enveloped viruses (Pfaender et al., 2013). This effect seems to be dependent on free fatty acids which are released upon storage by milk lipases in a time-dependent manner and which incorporate into the viral envelope thereby destroying viral integrity (Pfaender et al., 2013). Likely, the same principle is true for the observed effects on ZIKV. Most importantly, this process mimics the natural digestion of breastmilk upon which free fatty acids are released in the stomach of the infant (Hamosh et al., 1985). Next to the direct antiviral effect of stored milk, Holder pasteurization (62.5 °C for 30 min) offers the opportunity to inactivate potential viral contaminants, while maintaining the important bioactive factors of milk. This includes the inactivation of human immunodeficiency virus, human T-lymphoma virus, and cytomegalovirus, but also tuberculosis and other bacterial contaminants (Tully et al., 2001). We tested the effect of pasteurization on ZIKV infectivity and found that virus was effectively inactivated by milk pasteurization. We thereby present an easy and cost-effective approach to inactivate potential infectious viral particles in the breastmilk to prevent a possible transmission via breastfeeding in cases of doubt.

Conflict of interest

The authors declare no conflict of interest.

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