Bovine lactoferrin activity against Chikungunya and Zika viruses
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Abstract
Chikungunya (CHIKV) and Zika (ZIKV) viruses are arboviruses which have recently broken their sylvatic isolation and gone on to spread rampantly among humans in some urban areas of the world, especially in Latin America. Given the lack of effective interventions against such viruses, the aim of this work was to evaluate the antiviral potential of bovine lactoferrin (bLf) in their infections. Through viability, plaque, immunofluorescence and nucleic acid quantification assays, our data show that bLf exerts a dose-dependent strong inhibitory effect on the infection of Vero cells by the aforementioned arboviruses, reducing their infection efficiency by up to nearly 80%, with no expressive cytotoxicity, and that such antiviral activity occurs at the levels of input and output of virus particles. These findings reveal that bLf antimicrobial properties are extendable to CHIKV and ZIKV, underlining a generic inhibition mechanism that can be explored to develop a potential strategy against their infections.

Over the past few years, the world has witnessed epidemics of human infections caused by two old acquaintance, yet still obscure, arboviruses: Chikungunya (CHIKV) and Zika (ZIKV) viruses. While CHIKV is a member of the genus *Alphavirus* in the family *Togaviridae*, first isolated in Tanzania, in 1952 [1, 2], ZIKV is a member of the genus *Flavivirus* in the family *Flaviviridae*, first isolated in Uganda, in 1947 [3, 4]. These viruses are mainly transmitted by mosquitoes belonging to the genus *Aedes*, and are the etiological agent of Dengue-like febrile illnesses that show a range of superimposing unspecific signals and symptoms [5]. Chikungunya fever is frequently associated with a high prevalence of chronic arthralgia and ZIKV may be associated with congenital microcephaly [6]. As for other arbovirus diseases, no effective antiviral intervention is hitherto available for cases of Chikungunya or Zika fevers [7].

In search of a double shot, broad-spectrum drugs from nature may shed light on potential targets [8]. Among these drugs, lactoferrin (Lf) – an iron-binding globular glycoprotein of about 700 amino acid residues belonging to the transferrin family [9] – is noteworthy. First isolated from bovine (bLf) and human (hLf) milk in 1960 [10, 11], Lf is also found in various mucosal secretions, such as tears, saliva and seminal/vaginal fluids, and in the secondary granules of mature neutrophils [12, 13], playing an important role in the primary defence against a broad spectrum of pathogenic micro-organisms, including bacteria, protozoa, fungi and many naked and enveloped viruses [14]. Lf is currently manufactured in large scale, and using this commercially available material has advanced scientific research on Lf applications from basic studies to clinical trials [15]. The aim of this work was to evaluate the antiviral potential of iron-unsaturated bLf (Life Extension, Fort Lauderdale, USA) in the infection of African green monkey kidney (Vero) cells (American Type Culture Collection, Manassas, USA) by Brazilian strains of CHIKV (BeH807658) or ZIKV (BeH815744) as a way to identify common events in their life cycles that are liable to inhibition.

In order to assess whether bLf treatment would lead to toxic effects in Vero cells, a viability assay based on the cleavage of the fluorogenic, cell-permeant, peptide substrate glycylphenylalanyl-aminofluorocoumarin (GF-AFC) was carried out after incubating the cells with a range of bLf concentrations for 48 or 96 h at 37 °C, using the CellTiter-Fluor Cell Viability Assay (Promega, Fitchburg, USA). In general, viability of cells exposed to bLf was retained to a large extent: even at the highest bLf concentration tested, i.e. 1.0 mg ml⁻¹, no significant cytotoxicity was detected after 48 h treatment and cell viability was still approximately 75 % after 96 h treatment (Fig. 1a).

Keywords: antiviral; arbovirus; bovine lactoferrin; Chikungunya virus; Zika virus.

Abbreviations: aa, amino acid; apolf, apolactoferrin; bLf, bovine lactoferrin; CHIKV, Chikungunya virus; FITC, fluorescein isothiocyanate; GF-AFC, glycylphenylalanyl-aminofluorocoumarin; hLf, human lactoferrin; holoLf, hololactoferrin; IC₅₀, half maximal inhibitory concentration; iLFA, indirect immunofluorescence assay; JEV, Japanese Encephalitis virus; Lf, lactoferrin; MAYV, Mayaro virus; m.o.i., multiplicity of infection; p.f.u., plaque-forming unit; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RNA, ribonucleic acid; SD, standard deviation; ZIKV, Zika virus.
antiviral activity, similarly preventing CHIKV or ZIKV infection by nearly 80% at a concentration of 1.0 mg ml\(^{-1}\) (Fig. 1b). However, the half maximal inhibitory concentration (IC\(_{50}\)) of bLf was 0.2±0.005 mg ml\(^{-1}\) for CHIKV and 0.4±0.006 mg ml\(^{-1}\) for ZIKV.

A time-of-addition assay was next performed to determine the steps in CHIKV or ZIKV infection inhibited by bLf. In this approach, 1.0 mg ml\(^{-1}\) bLf was incubated with Vero cells before, during or after virus addition, and then tested as above for its effects on plaque formation. For both viruses, it was observed a significant antiviral activity of bLf at two of the three time points tested – before or during virus addition for CHIKV and during or after virus addition for ZIKV (Fig. 2a). Nevertheless, this inhibitory effect was clearly more pronounced when the protein was present together with the viruses, preventing CHIKV infection by approximately 70% and ZIKV infection by approximately 75%. When bLf was present before virus addition, it significantly inhibited CHIKV (approximate inhibition of 25%) but not ZIKV; inversely, when the protein was present after virus addition, it significantly inhibited ZIKV (approximate inhibition of 60%) but not CHIKV.

Given the large inhibitory effect promoted by bLf when it was present together with the viruses, an assay to evaluate its direct effect on CHIKV or ZIKV particles was carried out. In this approach, viruses were pretreated with 1.0 mg ml\(^{-1}\) bLf for 1 h at 37°C and titrated by plaque assay [16] in Vero cells after serial dilutions of virus samples, which reduced the concentration of bLf far below the minimum inhibitory concentration. CHIKV or ZIKV pretreatment with 1.0 mg ml\(^{-1}\) bLf for 1 h at 37°C showed no significant effects on virus infectious titres (Fig. 2b).

Since it seemed clear that bLf was mostly inhibiting an early event in the virus life cycle, the protein was tested for its ability to impair infection by interfering with virus binding/entry. In this experiment, Vero cells were first treated with 1.0 mg ml\(^{-1}\) bLf at 4°C to retain protein molecules at the cell surface and then briefly incubated with CHIKV or ZIKV at the same temperature after washing away unbound protein molecules, being afterwards washed again to remove unbound virus particles and incubated at 37°C to allow for infection progress. As assessed by indirect immunofluorescence assay (iIFA) using homemade anti-CHIKV or anti-ZIKV primary mouse polyclonal antibodies, obtained from the ascitic fluid of Swiss mice after intraperitoneal inoculations of live viruses as described elsewhere [17], and FITC-conjugated anti-mouse IgG secondary goat polyclonal antibodies (Sigma-Aldrich), bLf-treated cells showed very low levels of infection for both viruses when compared to mock-treated cells (Fig. 3a). For CHIKV, 31.2% of the cells were infected when mock-treated and 4.4% of the cells were infected when bLf-treated (~sevenfold inhibition); for ZIKV, 37.6% of the cells were infected when mock-treated and 9.7% of the cells were infected when bLf-treated (~fourfold inhibition).

Given the lack of cytotoxicity in the range of 0.2 to 1.0 mg ml\(^{-1}\), bLf was assayed for its antiviral potential in CHIKV or ZIKV infection in Vero cells under these concentrations. In such assay, bLf was incubated along the whole infection procedure, including a pretreatment step for 1 h at 37°C, and its ability to promote plaque number reduction was then tested. bLf showed a remarkable dose-dependent antiviral activity, similarly preventing CHIKV or ZIKV infection by 60% at a concentration of 0.2 mg ml\(^{-1}\), and its ability to promote plaque number reduction was significant (**, P<0.01). (b) Monolayers of Vero cells were incubated with the indicated concentrations of bLf at 37°C throughout the course of infection by CHIKV (black) or ZIKV (grey), including immediately before (for 1 h), during (for 1 h) and immediately after (for 48 or 96 h, respectively) virus addition under the same m.o.i. Cells were stained and plaques were counted to determine the efficiency of infection. Data were obtained from four to six experiments and plotted as mean±SD along with their exponential fittings, which revealed bLf IC\(_{50}\) values of 0.2±0.005 mg ml\(^{-1}\) for CHIKV and 0.4±0.006 mg ml\(^{-1}\) for ZIKV. All differences compared to the respective controls were significant (P<0.001).
Cells were first incubated with CHIKV or ZIKV at 37°C, allowing for the entry of virus particles into the cell and then briefly treated with 1.0 mg ml\(^{-1}\) bLf at the same temperature after washing away unbound virus particles, being afterwards washed again to remove unbound protein molecules and incubated at 37°C to allow for infection progress. As assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the SuperScript III Platinum One-Step qRT-PCR Kit with ROX (Invitrogen, Carlsbad, USA) in addition to previously described primer/probe sets (Integrated DNA Technologies, Coralville, USA) against defined sequences in CHIKV or ZIKV genomes [18, 19], the supernatant of the bLf-treated cell culture showed approximately half of the virus load for both viruses when compared to the supernatant of the mock-treated cell culture (Fig. 3b).

Despite much in evidence, CHIKV and ZIKV are just a minimal fraction of the myriad of arboviruses that may emerge or re-emerge in tropical and temperate regions of the world, especially in the Americas [20]. However, no selective inhibitors are available for a multitude of (re) emerging medically important viruses – in this scenario, broad-spectrum antiviral agents such as bLf, may offer important clues to cope with the challenge [21].

This study investigated whether the antiviral properties of bLf may be extended to CHIKV or ZIKV infection in Vero cells. Our data revealed a dose-dependent strong inhibitory effect by the protein in both cases, with no expressive cytotoxicity, reaching a similar maximum inhibition of nearly 80% at 1.0 mg ml\(^{-1}\) via different IC\(_{50}\) values (~0.2 mg ml\(^{-1}\) for CHIKV and ~0.4 mg ml\(^{-1}\) for ZIKV). Previous studies using bLf against a different emerging alpha – Mayaro virus (MAYV) – or flavivirus – Japanese Encephalitis virus (JEV), demonstrated slightly higher IC\(_{50}\) values (~0.4 and ~0.5 mg ml\(^{-1}\), respectively) in comparison to the respective virus counterparts addressed in this work [22, 23]. Such a difference indicates that CHIKV and ZIKV may be a little more sensitive than MAYV and JEV, respectively, to the effects of bLf.

The inhibitory activity of bLf over CHIKV or ZIKV infection was mostly exerted at a pre-entry step in virus infection (presumably binding/entry), but the protein also affected a post-entry step in this process (presumably production/exit). However, since treatment of virus particles with bLf before infection did not significantly affect their infectivity, its antiviral effect was not due to direct interaction with virus particles – i.e. bLf was not virucidal. Based on previous studies, the effects of Lf on virus binding/entry might be explained by the blockage of cell-surface glycosaminoglycans such as heparan-sulfate, exploited by many virus species as an unspecific adhesion molecule [24], while the effects of Lf on virus production/exit might be explained by its RNase activity, which could lead to virus RNA degradation [25].

It is worth noting that the observations derived from the iIFA and qRT-PCR experiments are not in contradiction with their counterparts derived from the time-of-addition experiment, as the analyses were performed under slightly different conditions by approaches that assess virus infection efficiency from different standpoints. While in the iIFA experiment bLf pre-treatment was carried out at 4°C, in the time-of-addition
experiment this procedure was carried out at 37 °C. Since both endocytosis and vesicle trafficking are active at 37 °C but not at 4 °C, the occurrence of only a slight antiviral effect in the time-of-addition experiment is probably associated with partial bLf internalization and fast glycosaminoglycan turnover, to which the protein is known to bind [26]. Regarding the comparison between the observations derived from the qRT-PCR and the time-of-addition experiments, it is important to bear in mind that post-entry events which only partially impair the virus infection process not necessarily lead to plaque number reduction, since even a minimal amount of virus progeny is able to promote the radial death zone that characterizes the plaque. Thus, as virus production/exit was not fully inhibited when bLf was added after virus entry, some virus progeny was still able to account for plaque formation in the time-of-addition experiment.

Although bLf has nearly 70 % amino acid sequence identity with hLf [27], the bovine version of the protein is often reported to exhibit higher antiviral activity than its human version [28]. Moreover, iron-unsaturated Lf (apoLf) is more potent than its iron-saturated isoform (holoLf) against some virus species [29]. Interestingly, Lf also contains various conserved peptides which are released upon its hydrolysis by proteases and still retain the antimicrobial activity [30]. bLf has been applied to commercial food products for the last couple of decades, and previous studies have demonstrated that its oral administration exerts a host-protective effect in various animals and in humans [31]. It is such a thermostable protein that its structure and antimicrobial properties are largely retained even after being subjected to pasteurization [32, 33].

When it comes to the delivery of protein drugs by oral administration, it is worth noting that, except in neonates for a few days after birth, intact proteins are usually not absorbed by enterocytes and there is virtually no absorption of peptides longer than 4 aa [34]. However, several studies have shown the presence of an intestinal receptor for Lf in both neonatal and adult animals, including humans, which is responsible for uptake of the molecule via transcytosis following oral administration [35]. In neonatal pigs, orally administered bLf appears in the blood circulation and reaches a peak level after 2 h [36]. In rats, the elimination half-life of intravenously administered bLf is approximately 30 min and the protein mainly accumulates in the liver, spleen and kidneys – furthermore, a high brain uptake is also observed [37]. Alternatively, Lf can also be delivered by intravenous administration in a nanoencapsulated formulation to improve stability and circulation time, without loss of activity [38]. Thus, an anti-arboviral strategy using Lf or their derivative peptides may rely on their enteral or parenteral administration, provided that an effective plasma concentration is achieved.
The risk of CHIKV and ZIKV adaptation to urban mosquito vectors other than Aedes aegypti and Aedes albopictus – such as Culex quinquefasciatus – due to the current rampant spreading of these viruses, especially in Latin America, may predict an even greater geographical dispersion of their respective diseases [39]. Added to this, the risk of CHIKV/ZIKV introduction in a new sylvatic environment – such as the Amazon rainforest – may establish permanent virus reservoirs for constant outbreaks in the newly affected areas, similar to the sylvatic cycle of yellow fever in Brazil [40]. Given the current scenario and these potential risks, there is an urgency for efficient prophylactic and therapeutic approaches against Chikungunya and Zika fevers. Our work shows that the antiviral properties of bLf are extendable to CHIKV and ZIKV and may be explored to design a two-in-one strategy against their infections.

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**Ethical statement**
This study made no use of human or vertebrate animal subjects and/or tissue.

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