Journal of Infectious Diseases and Therapy
ISSN: 2332-0877

Jones and Okeoma, J Infect Dis Ther 2015, 3:4
DOI: 10.4172/2332-0877.1000226

Research Article Open Access

Detection of Chikungunya virus (CHIKV) in urine of infected mice: a Potential Non-invasive Diagnostic Tool for CHIKV

Jones PH[1] **and Okeoma CM**[1,2*]

1Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

2Interdisciplinary Graduate program in Molecular and Cellular Biology (MCB), University of Iowa, Iowa City, IA, USA

[*]**Corresponding Author**: Chioma M Okeoma, Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, USA, Tel:319-335-7906; E-mail: chioma_okeoma@uiowa.edu

Received date: June 3, 2015; **Accepted date:** August 1, 2015; **Published date:** August 8, 2015

Abstract

Background: The reemergence of Chikungunya virus (CHIKV) infection and the spread of the virus throughout the world call for a fast, reliable, and improved laboratory diagnostic procedures. The aim of this study was to determine if CHIKV genome is present in other body fluids besides plasma as a tool for developing non-invasive diagnostic method.

Methods: This study utilized a sensitive and less invasive assay that combines the specificity of immuno-complexing and the sensitivity of real-time PCR to detect CHIKV in urine and plasma of infected mice.

Results: CHIKV RNA was detected by immuno-capture-quantitative reverse transcriptase PCR (IC-qPCR) in urine and plasma of infected mice. Viral RNA was detected in urine up to day 30, long after viral clearance in plasma. Furthermore, infectious CHIKV was detected in urine by cell-based immunofluorescence and 50% tissue culture infective dose ($TCID_{50}$) assays, suggesting the presence of infectious virus in urine.

Conclusion: We demonstrate a potential non-invasive diagnostic approach that could be adapted to screen for CHIKV infection in viremic and aviremic patients using urine.

Keywords Chikungunya; CHIKV; Urine; Plasma; Immuno-capture PCR

Introduction

Chikungunya virus (CHIKV) infections predominantly occur in tropical and subtropical areas of the world. However, in 2013, CHIKV was reported in the Americas, including 2014 report of local transmission in the United States. At the time of writing this manuscript in December of 2014, the number of traveler-associated CHIKV cases in US states and territories stand at 1,911 cases from US, 32 in Puerto Rico, and 6 in US Virgin Islands. The number of locally-transmitted cases stands at 11 from the state of Florida (US), 3,402 in Puerto Rico, and 86 in US Virgin Islands, (http://www.cdc.gov/chikungunya/geo/united-states.html).

With the rapid and continued spread of CHIKV outside and within the US, it is imperative to understand the pathogenesis of CHIKV and to develop a reliable non-invasive method of detection and/or diagnosis of CHIKV. Infection with CHIKV results in a spectrum of clinical symptoms, such as fever, encephalitis, neuropathy, and myelopathy [1-3]. Urine retention and paraparesis have also been associated with CHIKV infection [3]. Compared to healthy controls, CHIKV-infected patients were shown to have high levels of proline, hydroxyproline, and mucopolysaccharides in their urine [4].

Most but not all sites of CHIKV replication and CHIKV-associated disease have been identified [5-10]. Infection with CHIKV is presumed to cause encephalitis, myelopathy and neuropathy and patients infected with CHIKV present with urinary symptoms [1-3]. However, it is unknown whether the urinary tract organs and tissues such as kidney and bladder are infected with CHIKV. This current research was conducted to assess whether CHIKV infects urinary tract organs and tissues and if CHIKV could be detected in the urine of infected hosts.

Materials and Methods

Virus stock and virus propagation

CHIKV strain 181/25 [5] was inoculated onto Vero cells. Upon appearance of cytopathic effects, culture supernatants were harvested, clarified (1,000 × g for 5 min), aliquoted, and stored (-80°C) until titres were determined by end point dilution assay on Vero cells.

Animals and cells

5-week old female C57BL/6 mice were purchased from NCI – Frederick National Laboratory and maintained at the University of Iowa vivarium. Mice were inoculated on the hind footpad with CHIKV 181/25 (1.5×10^7 PFU) as previously described [6] and according to protocol approved by the University of Iowa Animal Care and Use Committee (IACUC). Vero cells were from the American Type Culture Collection (ATCC)]. Cells were maintained in Dulbecco's modified Eagle's medium as recommended by the ATCC.

Antibodies

Mouse anti-CHIKV was obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) through Dr. Robert Tesh of the University of Texas Medical Branch (UTMB) Galveston, Texas. Alexa fluor goat anti-mouse secondary antibody was from Life Technology.

Sample collection

Matched specimens (plasma, urine, kidney, and bladder) were collected from infected mice. Matched longitudinal plasma and urine samples were collected and evaluated. Following collection, samples were processed and stored at -80°C until required for analysis.

Detection of infectious CHIKV

Serial dilutions of infectious fluids were inoculated onto replicate Vero cells using a previously published protocol [6]. Cells were examined for cytopathicity and the end point (day 5 for our assays) was calculated from the data and expressed as 50% tissue culture infective dose ($TCID_{50}$). Alternatively, infectious virus was determined by confocal microscopy of Vero cells plated on cover slips.

Confocal microscopy

Vero cells infected with CHIKV were stained with anti-mouse CHIKV polyclonal antibody and counterstained with the appropriate secondary antibody [6,7]. Confocal images were acquired using Zeiss 510 confocal microscope [8].

RNA isolation, viral and host gene mRNA quantification

Total RNA was isolated from infected specimens using RNeasy mini kit (Qiagen). Equivalent amounts of DNase I (Qiagen) treated RNA were reverse-transcribed with high capacity cDNA reverse transcription Kit (ABI). Target specific primers including CHIKV nsP1 and E1 sequences were amplified using 7500 fast real-time PCR machine (ABI) as previously described [8-13].

Immuno-capture and Viral RNA quantification by PCR

200 µl of anti-CHIKV antibody diluted 1:100 in 50 mM NaCO3 (pH 9.6) was added into 0.2 ml thin-wall tubes (TempAssure PCR 8-tube strips with caps, USA Scientific) and incubated for 4 h at 37°C. The tubes were emptied and replaced with 1% bovine serum albumin in 50 mM NaCO3 (pH 9.6) to block unspecific binding. Tubes were incubated at 37°C for 1 h followed by thorough washing (three times) with buffer containing PBS, 0.05% Tween, and 0.02% sodium azide. After washing, 2 µl of clarified urine or plasma that are free of cellular debris were diluted in PBS to a total volume of 200 µl. Diluted samples were added to the tubes and incubated at 4°C overnight. Tubes were thoroughly washed (six times) with buffer containing 50 mM KCl, 10 mM Tris (pH 9), and 1.2 mM $MgCl_2 \bullet 6H_2O$. Captured virions were incubated in a buffer containing 50 mM KCl, 10 mM Tris (pH 9), 1.2 mM $MgCl_2 \bullet 6H_2O$, and 0.1% Triton X-100 for 5 minutes at room temperature. Triton X-100 was used to directly lyse the virus circumventing the need for RNA extraction. The immunocomplex was used for cDNA synthesis by the addition of cDNA synthesis reagents (high-capacity cDNA kit, Applied Biosystems) followed by PCR detection of relative CHIKV E1 expression by real-time PCR. The following primers were used: E1F: GCATCAGCTAAGCTCCGGGTC and E1R: CAATGTCTTCAGCCTGGACACC [14].

Statistics

Statistical analysis was performed by paired Student's t-test, and data presented as mean ± standard deviation (SD). Error bars represent standard deviations and significance was taken at $P<0.05^{*}$ or $<0.01^{**}$.

Results

CHIKV infects bladder and kidney

To determine whether the kidney and/or bladder are susceptible to CHIKV, we collected the kidneys and bladders from 5-week old female C57BL/6 mice that were infected with live-attenuated CHIKV 181/25. To assess the extent of CHIKV gene expression in kidney and bladder, we extrapolated E1 (Figure 1A) and nsP1 (Figure 1B) abundance from standard curves generated from dilution series of E1 or nsP1 amplified from CHIKV infected Vero cells. CHIKV gene expression values were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that was used as an endogenous control to correct for sample to sample variations [6]. Although variability exists among infected mice tissues, more CHIKV RNA was detected in the kidney compared to the bladder. Furthermore, we evaluated the level of infectious virus present in the kidney and bladder. Interestingly, both kidney and bladder produced infectious virus as revealed by $TCID_{50}$ experiments (Figure 1C). These results suggest that CHIKV infects, replicates, and produces infectious virus in the kidney and bladder.

Immuno-capture-quantitative reverse transcriptase PCR (IC-qPCR) based detection of CHIKV RNA in urine samples

The mammalian bladder collects and stores urine excreted by the kidneys. Because the kidney and bladder are susceptible to CHIKV and infectious virus is present in these tissues, we queried whether CHIKV is shed in urine of infected mice.

Immuno-capture of CHIKV particles shed into urine samples of infected mice was performed with anti-CHIKV polyclonal antibody followed by PCR amplification of CHIKV E1 sequences. PCR amplicons were separated by agarose gel electrophoresis and visualized with ethidium bromide as previously described [15,16].

Results show that CHIKV genome is present in both plasma and urine of infected mice (Figure 2A). Comparative analysis of CHIKV RNA in plasma and urine by quantitative PCR reveals a significant difference between plasma and urine CHIKV RNA content (Figure 2B).

Limit of CHIKV RNA detection by Immuno-capture-quantitative reverse transcriptase PCR (IC-qPCR)

To determine the limit of CHIKV detection by IC-qPCR, we generated E1 standard curve as described in figure 1. The standard curve was used to extrapolate E1 copy numbers from a series of dilutions constructed from cell free CHIKV isolated from infected Vero cells. The limit of detection—defined as the minimum concentration at which IC-qPCR distinguished a positive sample from negative sample is approximately 2 copies/ µl (Figure 2C).

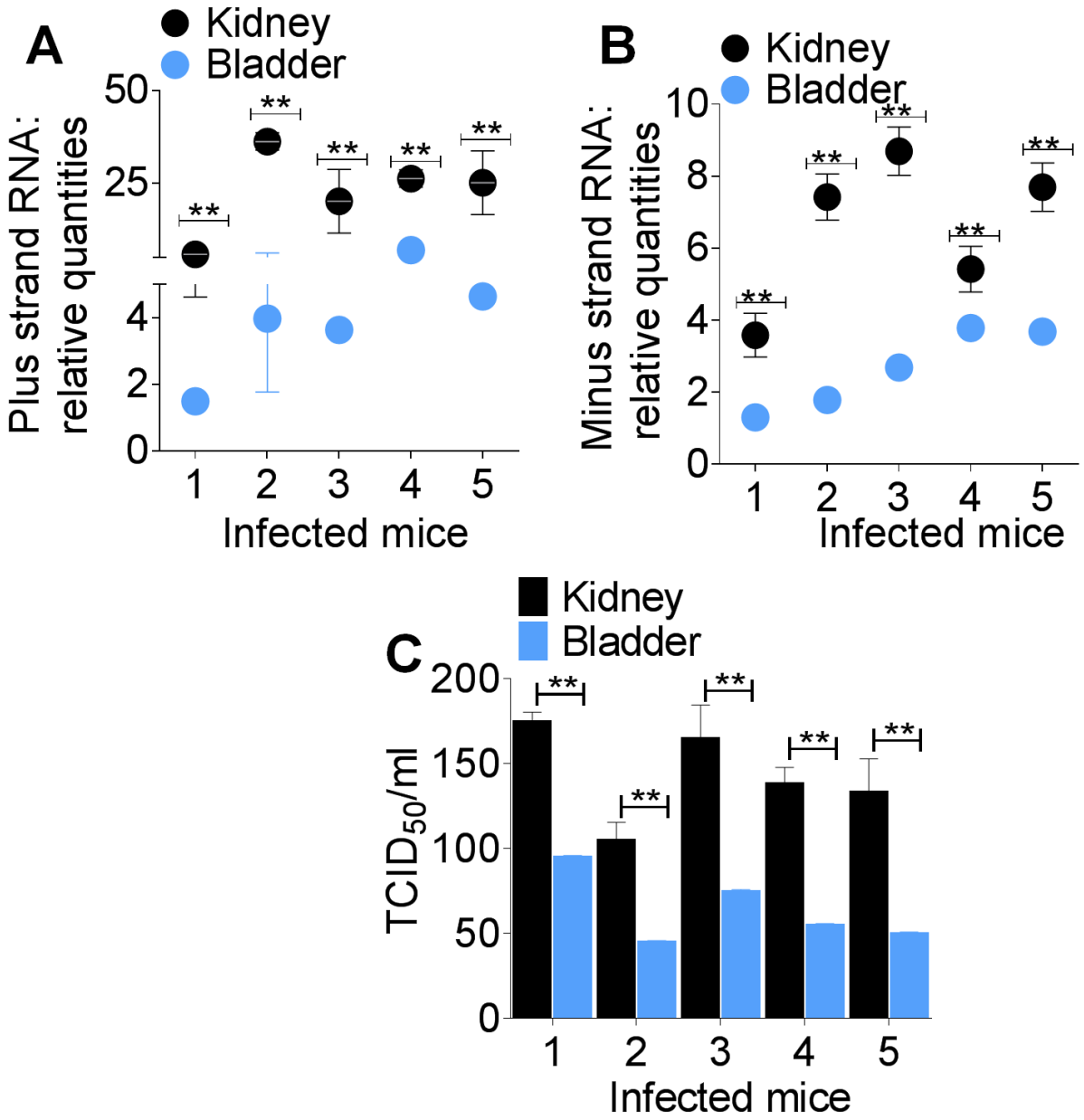

Figure 1: CHIKV infects murine kidney and bladder: A, B, 5-week old female C57BL/6 mice (n=5) were inoculated with CHIKV 181/25 subcutaneously on the hind footpads. Viral load in the kidney and bladder were determined by quantitative real-time PCR (RT-qPCR) 24 h after infection using the standard curve method. Relative quantities of CHIKV plus and minus strand RNAs were extrapolated from standard curves generated from dilution series of E1 (plus strand) or nsP1 (minus strand) amplified from CHIKV infected Vero cells. PCR data were normalized to GAPDH used as internal control. C, equivalent mass of kidney and bladder tissues were homogenized in medium. Clarified tissue lysates were used to inoculate Vero cell monolayers for $TCID_{50}$ studies. Error bars are standard deviations and significance was taken at $P<0.01^{**}$. Experiments were repeated three times with similar results.

Comparison of CHIKV detection rates between plasma and urine samples

To further understand the differences between plasma and urine viral loads by IC-qPCR, we used autologous serial plasma and urine samples collected from infected mice. Naïve samples were used as controls. CHIKV was present in urine samples on all days tested (days 1, 3, 5, 7, 9, and 30—experimental end point) with some variability in the amount of virus detected on a given day (Figure 2D).

However, plasma samples were positive for CHIKV on days 1 to 7. By day 9, plasma viral RNA has diminished below day 1 level, while urine viral RNA peaked (Figure 2D), suggesting that viral shedding into urine may be occurring.

Although declining, viral load in urine remained high up to day 30 post infection. These results reveal that each sample type could be of use in detecting CHIKV. However, urine sample has more value as CHIKV could be detected in urine long after the virus is cleared from the blood. Therefore, detection and quantitation of urine-associated viral load by IC-qPCR may provide a reliable and non-invasive method of CHIKV detection.

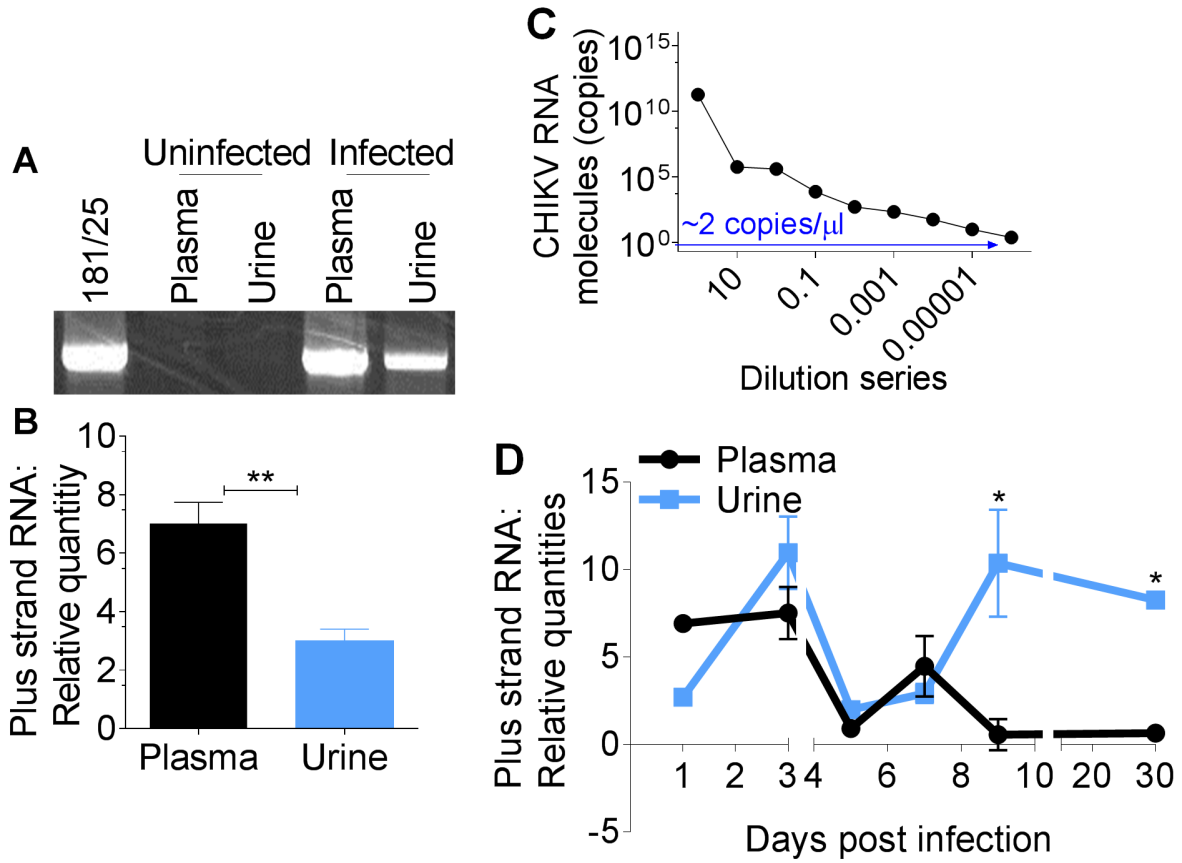

Figure 2: Presence of CHIKV RNA in urine: A, B, Matched cell-free plasma and urine samples collected from infected mice (n=5) were subjected to immuno-capture and quantitative PCR (IC-qPCR) detection of CHIKV plus strand RNA. A, Amplicons were separated by agarose gel electrophoresis and stained with ethidium bromide for visualization of CHIKV RNA. CHIKV 181/25 collected from Vero cell culture was used as positive control. Plasma and urine from uninfected mice were used as negative controls. B, IC-qPCR analysis of CHIKV RNA using the standard curve method. C, Limit of CHIKV detection by IC-qPCR was determined using standard curve constructed from a series of dilutions of CHIKV E1 RNA amplified from cell free CHIKV. D, Viral load in matched plasma and urine were detected over time by IC-qPCR using the standard curve method. Relative quantities of CHIKV plus RNA was extrapolated from a dilution series of E1 RNA amplified from CHIKV infected Vero cells. Data were normalized to GAPDH which was used as internal control. Error bars are standard deviations and significance was taken at $P<0.05^{*}$; ns: Not significant. Experiments were repeated three times with similar results.

Detection of infectious CHIKV in urine samples

Presence of infectious CHIKV in urine was assessed by immunofluorescent (IF) detection of CHIKV antigen and $TCID_{50}$ studies. Infectious virus was detected by IF in Vero cells inoculated with plasma and urine collected from mice 24 h after infection. Cells were stained with anti-mouse CHIKV polyclonal antibody [6] and confocal images were acquired using Zeiss 510 confocal microscope. While robust CHIKV antigen was detected in Vero cells inoculated with plasma, a little above background antigen staining was present in cells inoculated with urine (Figure 3A).

Alternatively, serial dilutions of infectious fluids were inoculated onto replicate Vero cells using a previously published protocol [6]. Cells were examined for cytopathicity to quantify virus titer using endpoint dilution assay [6], and results were expressed as $TCID_{50}$. About 2×10^{2} CHIKV particles/ml was detected in Vero cells inoculated with plasma compared to 1×10^{-1} particles/ml present in urine-inoculated Vero cells (Figure 3B). These results indicate that while comparable levels of CHIKV genome may be present in both urine and plasma, less infectious particles reside in urine.

Discussion

Outbreaks of CHIKV infections have occurred throughout the world since its discovery in 1952. Infected persons present with acute febrile illness associated with mild to severe skin rash, joint pain (Arthritis) and muscle pain (Myalgia) that can persist long after virus clearance, resulting in high morbidity. Renal dysfunction has also been

linked to CHIKV infection [3] but whether the urinary tract is susceptible to CHIKV infection is unknown.

In this study, we reveal that CHIKV infects the bladder and kidney of the host and that CHIKV RNA is present in urine of infected mice. Interestingly, infectious CHIKV was also detected in the bladder and kidney, as well as in urine samples. However, the titre of urine-associated infectious virus was significantly lower compared to plasma-associated virus from the same mice. It is not surprising that the titre of infectious CHIKV in urine is extremely low since other mosquito borne viruses such as West Nile Virus [17] and Dengue virus [18] have detectable viral genome in the absence of infectious virus in the urine of infected patients.

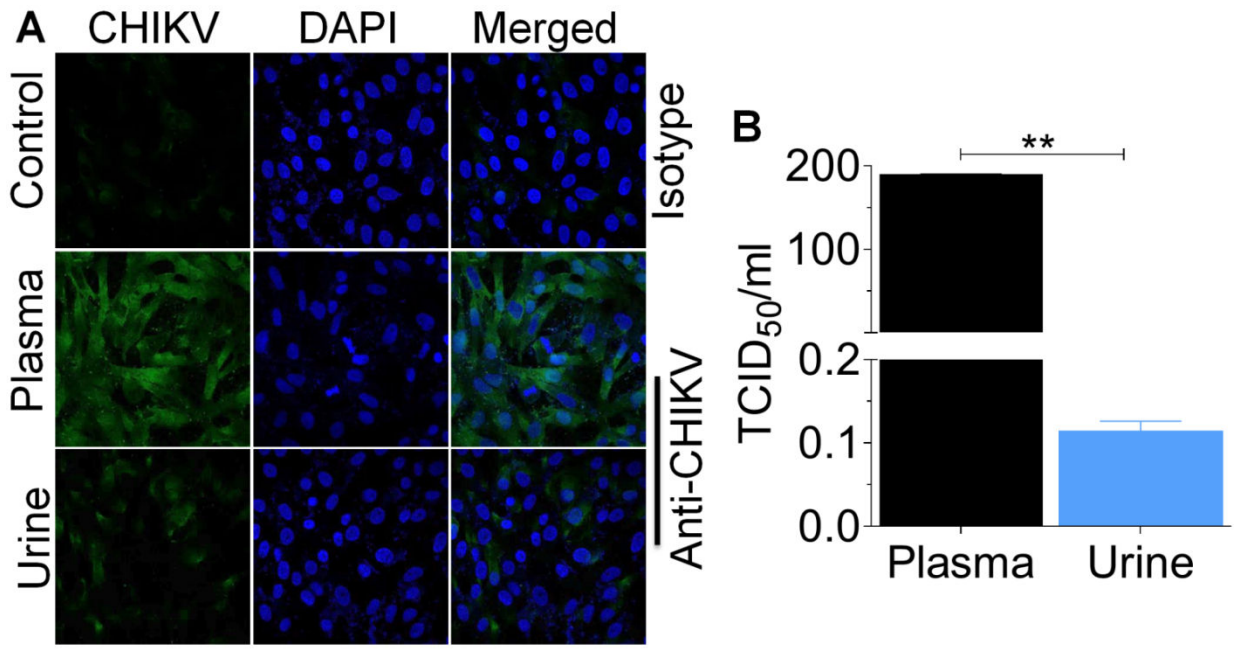

Figure 3: Detection of infectious CHIKV: A, Confocal imaging of CHIKV expression in Vero cells inoculated with equivalent volume of matched cell-free urine (top and bottom panels) or plasma (middle panel). Cells were stained with isotype control (top panel) or anti-CHIKV antibodies (middle and bottom panels). CHIKV is in green and DAPI (blue) stains the nucleus. B, Infectious CHIKV titer in matched plasma and urine of infected mice was determined by end point dilution analysis (EPDA) (6) and presented as $TCID_{50}$. Error bars are standard deviations and significance was taken at P<0.01**. Experiments were repeated three times with similar results.

The occurrence of CHIKV minus strand RNA and infectious virus in urine samples is indicative of low but active viral replication in the urinary tract. Replication of CHIKV in the kidney and bladder could be the pathological mechanism for the presence of CHIKV RNA in urine. Presence of CHIKV RNA in urine beyond 7 days meant that CHIKV diagnosis could still be made even during the aviremic stage of infection.

Conclusion

Presently, it is not known whether human urinary tract organs and tissues are susceptible to CHIKV infection. This study reveals active CHIKV replication in the kidney and bladder of mice and highlights the usefulness of urine for detection of CHIKV infection long after clearance of plasma viremia. Future studies should elucidate whether human urinary tract organs and tissues are susceptible to CHIKV and evaluate the usefulness of human urine as an additional and/or alternative non-invasive laboratory diagnostic tool for CHIKV infection.

Acknowledgment

This work was supported by Funds from the Department of Microbiology of the University of Iowa. Publication of this manuscript was made possible through core services from the University of Iowa Central Microscopy Research Facility. We thank Alexander Canfield and Bryson Okeoma of University of Iowa for help with endpoint dilution assay and constructive criticisms respectively.

References

1. Chandak NH, Kashyap RS, Kabra D, Karandikar P, Saha SS, et al. (2009) Neurological complications of Chikungunya virus infection. Neurol India 57: 177-180.
2. Rampal, Sharda M, Meena H (2007) Neurological complications in Chikungunya fever. J Assoc Physicians India 55: 765-769.
3. Baishya R, Jain V, Ganpule A, Muthu V, Sabnis RB, et al. (2010) Urological manifestations of Chikungunya fever: A single centre experience. Urol Ann 2: 110-113.
4. Lokireddy S, Vemula S, Vadde R (2008) Connective tissue metabolism in chikungunya patients. Virol J 5: 31.
5. Levitt NH, Ramsburg HH, Hasty SE, Repik PM, Cole FE Jr, et al. (1986) Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine 4: 157-162.

6. Mahauad-Fernandez WD, Jones PH, Okeoma CM (2014) Critical role for bone marrow stromal antigen 2 in acute Chikungunya virus infection. J Gen Virol 95: 2450-2461.
7. Jones PH, Maric M, Madison MN, Maury W, Roller RJ, et al. (2013) BST-2/tetherin-mediated restriction of chikungunya (CHIKV) VLP budding is counteracted by CHIKV non-structural protein 1 (nsP1). Virology 438: 37-49.
8. Jones PH, Mehta HV, Maric M, Roller RJ, Okeoma CM (2012) Bone marrow stromal cell antigen 2 (BST-2) restricts mouse mammary tumor virus (MMTV) replication in vivo. Retrovirology 9: 10.
9. Jones PH, Okeoma CM (2013) Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated BST-2/tetherin regulation. Cell Signal 25: 2752-2761.
10. Mehta HV, Jones PH, Weiss JP, Okeoma CM (2012) IFN-Î± and lipopolysaccharide upregulate APOBEC3 mRNA through different signaling pathways. J Immunol 189: 4088-4103.
11. Jones PH, Mahauad-Fernandez WD, Madison MN, Okeoma CM (2013) BST-2/tetherin is overexpressed in mammary gland and tumor tissues in MMTV-induced mammary cancer. Virology 444: 124-139.
12. Madison MN, Roller RJ, Okeoma CM (2014) Human semen contains exosomes with potent anti-HIV-1 activity. Retrovirology 11: 102.
13. Mahauad-Fernandez WD, DeMali KA, Olivier AK, Okeoma CM (2014) Bone marrow stromal antigen 2 expressed in cancer cells promotes mammary tumor growth and metastasis. Breast cancer res 16: 493.
14. Pongsiri P, Praianantathavorn K, Theamboonlers A, Payungporn S, Poovorawan Y (2012) Multiplex real-time RT-PCR for detecting chikungunya virus and dengue virus. Asian Pac J Trop Med 5: 342-346.
15. Okeoma CM, Stowell KM, Williamson NB, Pomroy WE (2005) Neospora caninum: quantification of DNA in the blood of naturally infected aborted and pregnant cows using real-time PCR. Exp Parasitol 110: 48-55.
16. Okeoma CM, Williamson NB, Pomroy WE, Stowell KM, Gillespie L (2004) The use of PCR to detect Neospora caninum DNA in the blood of naturally infected cows. Vet Parasitol 122: 307-315.
17. Tonry JH, Brown CB, Cropp CB, Co JK, Bennett SN, et al. (2005) West Nile virus detection in urine. Emerg Infect Dis 11: 1294-1296.
18. Hirayama T, Mizuno Y, Takeshita N, Kotaki A, Tajima S, et al. (2012) Detection of dengue virus genome in urine by real-time reverse transcriptase PCR: a laboratory diagnostic method useful after disappearance of the genome in serum. J Clin Microbiol 50: 2047-2052.