Published in final edited form as:

Virus Res. 2018 August 02; 254: 1–9. doi:10.1016/j.virusres.2017.07.011.

Mosquito-borne and sexual transmission of Zika virus: Recent developments and future directions

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Abstract

Zika virus (ZIKV; Genus *Flavivirus*, Family *Flaviviridae*) has recently emerged in Asia and the Americas to cause large outbreaks of human disease. The outbreak has been characterized by high attack rates, birth defects in infants and severe neurological complications in adults. ZIKV is transmitted to humans by *Aedes* mosquitoes, but recent evidence implicates sexual transmission as playing an important role as well. This review highlights the transmission of ZIKV in humans, with a focus on both mosquito and sexually-transmitted routes and their outcomes. We also discuss critical directions for future research.

Introduction

Zika virus (ZIKV) is currently causing a worldwide pandemic that likely began on the Micronesian island of Yap in 2007, but gained worldwide attention after spreading to Northeastern Brazil in 2013 or 2014 [1, 2]. ZIKV then rapidly spread through Brazil and subsequently throughout South and Central America, the Caribbean and caused a small outbreak in the United States [3]. ZIKV, like other human-infecting flaviviruses, normally exists in an arthropod to vertebrate host transmission cycle. Surprisingly, ZIKV has been shown to also be transmissible via the sexual route [4]. Infectious virus can be detected in semen and vaginal secretions, along with other body fluids [5–7]. ZIKV, therefore, has the unusual capacity (among arboviruses) to persist, in at least a limited manner, as a sexually transmitted infection (STI). This unique attribute of ZIKV, along with severe disease manifestations in infants and adults, has led to increased interest in understanding the virus and its transmission. ZIKV has been isolated from many species of mosquitoes, but *Aedes aegypti* appears to be the major vector for urban transmission in the Americas [8]. Outbreaks

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in urban areas of Oceania and Africa and sylvatic spread in Africa have been associated with other *Aedes* species [9, 10]. Sexual transmission of ZIKV has been reported in most affected countries and has been documented in animal models [4, 5, 11]. The contribution of each transmission cycle to virus perpetuation and disease will be discussed.

Mosquito Transmission of Zika virus

ZIKV Phylogeny

Flavivirus Phylogeny Relating to Transmission—Flaviviruses, including flavi-like viruses, include viruses that replicate in vertebrates only, arthropods only, plants only, and non-arthropod invertebrates in addition to those that replicate in a transmission cycle involving both vertebrates and arthropods (arboviruses) [12]. However, the main mode of transmission of disease-causing flaviviruses is via the bite of an infected arthropod. These viruses cluster phylogenetically according to the main vector [13]. In alignments of full-genome amino acid sequences of flaviviruses, ZIKV clusters within the mosquito-borne flaviviruses (MBFV) and more specifically within the group of *Aedes*-borne flaviviruses (ABFV) (reviewed in [14]), consistent with the historical literature on ZIKV that strongly suggested transmission by these mosquitoes.

Natural Transmission

Sylvatic Transmission—In the last ~70 years, ZIKV has been isolated from a wide array of mosquitoes during surveillance activities for mosquito-borne diseases. Consistent with its phylogenetic position within the ABFV group of flaviviruses, the first mosquito isolation of ZIKV was from a pool of *Ae. (Stegomyia) africanus* in the Zika forest in Uganda in 1948 [15]. Prior to this, ZIKV was isolated from a sentinel rhesus monkey in the same forest, suggesting mosquito transmission [15]. *Ae. africanus* are known to bite both at ground level and in the canopy of the Zika forest, and feed on both humans and monkeys and therefore represent a good sylvatic vector for ZIKV [16]. ZIKV was subsequently collected from this species in 1956 in Lunyo forest, Uganda, close to the Zika forest [17]; from *Ae. (Stegomyia) luteocephalus* in Nigeria [18] and *Ae. africanus/Ae. (Stegomyia) apicoargenteus* from Uganda, both in 1969 [19]. This study also identified several forest dwelling non-human primate (NHP) species that had serological evidence of ZIKV infection; including the red-tailed monkey (*Cercopithecus ascunius schmidtii*), black mangabey (now the grey checked mangabey) (then: *Cercocobus albigena johnstoni*, now: *Lophocebus albigena*), and the lowland colobus (now the Western Guereza) (then: *Colobus abyssinicus uellensis*, now: *Colobus guereza*) [19]. A 1969 report also references “strong serologic evidence” of ZIKV in wild monkeys in Malaysia, although they do not provide specific details [20]. ZIKV was then isolated again from *Ae. africanus* and also *Ae. (Stegomyia) opok* in the Central African Republic (CAR) between 1976–80 [21]. In 1984, mosquito surveys in Senegal identified ZIKV in both *Ae. africanus* and *Ae. (Diceromyia) taylori* mosquitoes [22]. Later surveys in Senegal between 1988–1991 found several more species positive for ZIKV; *Ae. (Diceromyia) furcifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. (Stegomyia) aegypti*, *Ae. (Stegomyia) neoaffricanus*, *Ae. (Aedimorphus) dalzieli*, *Ae. (Aedimorphus) fowleri*, *Ae. (Aedimorphus) minutus*, and *Ae. (Fredwardsius) vittatus*. Serological studies referenced in several papers also suggest that high levels of antibodies against ZIKV were found in NHPs,

although individual species were not mentioned [23, 24]. ZIKV was further isolated from *Ae. vittatus*, *Ae. furcifer*, and *Ae. aegypti* pools in the Ivory Coast in 1999 [25]. A large mosquito surveillance effort in Senegal in 2011 found many *Aedes* species positive for ZIKV, including; *Ae. furcifer*, *Ae. luteocephalus*, *Ae. africanus*, *Ae. vittatus*, *Ae. taylori*, *Ae. dalzieli*, *Ae. (Aedimorphus) hirsutus* and *Ae. (Stegomyia) metallicus*, *Ae. aegypti*, *Ae. (Stegomyia) unilineatus* [26]. This survey also found other mosquitoes positive for ZIKV in the subfamily Culicinae, namely *Mansonia (Mansonioides) uniformis*, *Culex (Culex) perfuscus*. Finally, ZIKV was isolated from a single pool of *Anopheles (Anopheles) coustani*. It must be determined if these species are capable of transmission or whether these isolations merely represent exposure to ZIKV by ingesting a bloodmeal from an infected animal.

Observational surveys of mosquitoes have thus implicated several arboreal mosquitoes, mainly in the genus *Aedes*, that tend to be involved in transmission of primate-hosted arboviruses like ZIKV. Because the vast majority of isolations of ZIKV occurred in the subgenera *Stegomyia*, *Aedimorphus*, *Diceromyia*, and *Fredwardsius*, these should be of particular interest in future surveillance studies. Many of the most ubiquitous arboviruses can be transmitted in sylvatic cycles by the same mosquito species. For example, *Ae. africanus*, *Ae. luteocephalus*, *Ae. furcifer*, *Ae. taylori*, *Ae. vittatus*, and *Ae. opok* have all been found positive for ZIKV, chikungunya virus (CHIKV), dengue virus (DENV) and yellow fever virus (YFV) [21, 24, 26–32]. Therefore, it is essential to maintain surveillance efforts of these mosquitoes and their associated NHP hosts to assess potential future arboviral threats.

The possibility that sylvatic transmission cycles could become established as ZIKV undergoes globalization has been addressed by a handful of studies, with some evidence of sylvatic transmission in Asia [33] and the Americas [34] reported. The report from the Americas finds two species of NHPs (n=7, *Sapajus libidinosus* and *Callithrix jacchus*) qRT-PCR positive in the serum. However, since low levels of RNA and no infectious virus was recovered, there is no direct evidence for sylvatic transmission being maintained. More sampling of NHPs throughout the Americas should be undertaken to assess this possibility. Some NHPs that were previously shown to be susceptible to yellow fever virus (YFV) in the Americas include the common squirrel monkey (*Saimiri sciureus*), the spider monkey (*Ateles ater*), the large-headed capuchin (*Sapajus microcephalus*) and to a lesser extent the brown woolly monkey (*Lagothrix lagotricha*) [35, 36]. These species in particular should be monitored for potential ZIKV infection given their relationship with YFV. The probability for a sustained sylvatic transmission cycle in the Americas remains unknown at this time and must be explored further. The closely related flaviviruses, DENV serotypes 1–4 and YFV have had very different success in establishing sylvatic cycles in the New World. The latter has a well-documented sylvatic transmission cycle involving mosquitoes in the genera *Haemagogus*, *Sabethes*, *Psorophora* and others (reviewed in [37]). In contrast, there is little evidence of a sylvatic DENV transmission cycle in the New World, despite some reports of infections in non-human primates and other animals [38–40]. Whether this is due to the host (either mosquito or NHP) or viral factors remains unknown. Studies should be undertaken to assess the possibility of ZIKV transmission by forest-dwelling mosquitoes of the New World

and whether associated NHPs are susceptible to infection, including the possibility of ZIKV maintenance by sexual transmission among NHPs.

Non-Sylvatic Transmission—Similar to other ABFVs, ZIKV has been mainly associated with transmission in urban settings by the highly anthropophilic mosquito *Aedes aegypti*, however several other species are thought to play a significant role as well. ZIKV was first isolated from *Ae. aegypti* in Malaysia in 1969 [20]. Further isolations of ZIKV in *Ae. aegypti* were reported in 1988–91 (and again in 2011) and 1999 in Senegal and the Ivory Coast, respectively [24–26]. However, in each case only one pool was found to be positive so the role in transmission may have been small. While *Ae. aegypti* is accepted as the dominant vector during the current outbreak in the Americas, few published reports of virus isolations from these mosquitoes exist. The first isolations of ZIKV from *Ae. aegypti* in the Americas came in 2015–16 from Mexico [41, 42] and Brazil [8]. In Brazil, the percent of mosquito pools positive for ZIKV was only 1.5% (3/198), while in Mexico up to 27.3% of pools were found positive (15/55). It's possible that surveillance done in Brazil, and previously in Malaysia, Senegal and Ivory Coast was done after the peak of the outbreak, thus underestimating the number of mosquitoes infected during peak transmission. In comparison, similar rates of DENV infection of *Ae. aegypti* have been observed both in Mexico (where multiple serotypes were circulating concurrently) and during an outbreak in Tanzania as were observed in Mexico for ZIKV [43, 44]. Therefore, infection rates of *Ae. aegypti* did not appear to be higher during the ZIKV outbreak than those previously observed for DENV.

Other *Aedes* species are thought to have been important for ZIKV epidemics, particularly recently. *Ae. (Stegomyia) albopictus* was the main vector for a ZIKV outbreak in Gabon in 2007 [10]. *Ae. albopictus* was also found naturally infected with ZIKV in Mexico [45]. Although not confirmed by virus isolation, *Ae. (Stegomyia) polynesiensis/Ae. aegypti* and *Ae. (Stegomyia) hensilli* were implicated as the predominant vectors in the outbreaks of French Polynesia and Yap Island, respectively [2].

Although ZIKV in Africa has been previously isolated from mosquitoes in the genera *Culex* and *Anopheles*, their role in transmission appears to be small based on previous field collections in which *Aedes spp.* are the predominant mosquitoes found infected [16, 26] and the lack of experimental evidence for their competence as vectors ([46, 47], see below). While no reports from the Americas have specifically looked for ZIKV in *Anopheles* mosquitoes, two reports have found no evidence for ZIKV in *Culex* mosquitoes in locations where *Ae. aegypti* have been found positive [8, 42]. Additional collections are necessary to determine the potential role of these mosquitoes in the transmission of ZIKV.

ZIKV Adaptation to Non-Sylvatic Transmission—Many previous arbovirus outbreaks have been associated with mutations that led to increased infectivity or transmission in mosquitoes that efficiently spread the arboviruses to humans. For example, the introduction of West Nile virus (WNV) into The United States in 1999 was associated with a valine to alanine change which resulted in a decreased extrinsic incubation period (EIP) in *Cx. pipiens* [48]. Similarly, an alanine to valine change in the E1 protein of CHIKV was previously shown to result in increased infectivity and transmission of *Ae. albopictus* which may have

resulted in the large outbreak on the French island of La Reunion and subsequent spread [49, 50]. It has recently been shown that an alanine to valine change in the NS1 of post-American epidemic ZIKV strains may be responsible for increased infectivity in *Ae. aegypti* mosquitoes [51]. Liu et al. show that the A188V mutation in NS1 is present in epidemic Asian genotype and African strains of ZIKV but not pre-epidemic Asian genotype strains, resulting in an increased amount of NS1 secreted from infected mammalian cells and immune deficient mice. This mutation, found in the Asian genotype strains after 2013, was associated with increased numbers of mosquitoes being infected after bloodfeeding, although they did not directly show that this resulted in increased transmission rates. This is consistent with previous reports showing that African strains of ZIKV are more fit in *Ae. aegypti* mosquitoes than even epidemic Asian genotype strains [47], suggesting additional genomic sites are likely to influence mosquito infectivity. Further studies are needed to assess the role of viral genetics in the American outbreak.

Experimental Transmission and Potential for Novel Vectors

Experimental Infection of Different Mosquito Species for ZIKV—The first report of experimental transmission studies came in 1956, when it was shown that *Ae. aegypti* mosquitoes from Nigeria fed an artificial bloodmeal could infect mice and rhesus monkeys [52]. It was later shown that ZIKV exposed *Ae. aegypti* also from Nigeria that were allowed to feed on a viremic human volunteer were unable to transmit to mice and were not found to be infected, suggesting little to no infectious virus in the mosquito body or saliva [53]. Presence of infectious circulating virus in the volunteer was confirmed by infections of mice with acute phase serum, however levels were low, as diluted serum was not infectious to mice. Following the outbreaks in Oceania and now the Americas, studies on mosquitoes' vector competence for ZIKV have risen sharply. *Ae. aegypti* from Singapore, French Guiana, Guadeloupe, Brazil, Martinique, Mexico, Australia, and Tahiti have all been shown to be capable of transmitting ZIKV after an artificial bloodmeal [47, 54–59]. In contrast, Senegalese *Ae. aegypti* from two locations were refractory to ZIKV transmission, as judged by lack of ZIKV RNA in the saliva [60]. The discrepancy between these studies is unclear, but may be due to differences in vector susceptibility in different locations. This highlights the importance of virus-mosquito interactions, even within the same species of mosquito. Most of the previous ZIKV isolations in Africa have been from species other than *Ae. aegypti*, suggesting that other *Aedes* vectors in that area may be more susceptible to ZIKV. Additional studies of ZIKV vector competence in African populations of *Ae. aegypti* with different ZIKV genotypes are required to examine the effect of virus genotype - mosquito population combinations on transmission outcome.

Various other *Aedes* species have been tested for their ability to transmit ZIKV. Those species that contained infectious virus in their saliva after ZIKV feeding include, *Ae. (Aedimorphus) vexans* [61], *Ae. albopictus* [54, 62–64], *Ae. vittatus*, and *Ae. luteocephalus* [60]. Species that were refractory to transmission include, *Ae. (Ochlerotatus) taeniorhynchus* [65], *Ae. (Stegomyia) unilineatus* [60], *Ae. (Ochlerotatus) triseriatus* [62], *Ae. (Finlaya) notoscriptus*, and *Ae. (Ochlerotatus) vigilax* [56]. *Ae. hensili* were found to be both body and head positive after ZIKV infection, but cannot be definitely called transmission positive

as the head could represent solely a disseminated infection without transmission potential [66].

In contrast to *Aedes*, most attempts at observing transmission or even infection in *Culex* and *Anopheles* mosquitoes have been unsuccessful. Both *An. (Cellia) gambiae* and *An. (Cellia) stephensi* were completely refractory to infection with ZIKV [46]. The overwhelming majority of attempts at infecting various species of *Culex* have proven unsuccessful. Species tested and found transmission negative include *Cx. (Culex) quinquefasciatus* [46, 47, 56, 65, 67–71], *Cx. (Culex) pipiens* [47, 62, 64, 67, 70, 72], *Cx. (Culex) tarsalis* [47], *Cx. (Culex) torrentium* [64], *Cx. (Culex) annulirostris* and *Cx. (Culex) sitiens* [56]. In contrast, a lone report of *Cx. quinquefasciatus* from China revealed high transmission rates as early as 8 days post-exposure [73]. The reasons for this discrepancy are unclear, but may be due to genetic factors associated with this species from the region. Studies should be repeated with mosquitoes from this region of China in order to validate this result. Additionally, since mosquitoes from only one subgenus for both *Anopheles* and *Culex* have been tested, additional species should be investigated as potential vectors.

Vertical Transmission of ZIKV—Two reports have confirmed the possibility of vertical transmission in *Ae. aegypti* mosquitoes [74, 75]. However, only one of these reports found *Ae. albopictus* to be capable of vertically transmitting ZIKV. It is possible that this difference was due to one study using intrathoracic injection [75] and the other using an infectious bloodmeal [74]. As the latter is more representative of an actual mosquito infection, it seems possible that vertical transmission could be a factor in ZIKV maintenance. In fact, ZIKV RNA was detected in field collected *Ae. albopictus* eggs [76]. Sustained vertical transmission would allow ZIKV to be maintained in the mosquito population without active human transmission, constituting a major risk for future ZIKV outbreaks. Additional studies in both the laboratory and the field are required to assess the potential for ZIKV to be maintained vertically in mosquitoes.

Future Directions

As ZIKV and other arboviruses continue to expand their range, it is imperative that measures to predict their spread proactively are implemented. Very little is known about the factors involved in arbovirus emergence. Likely candidates are viral evolution (as shown by the A188V NS1 mutation), host evolution (particularly the arthropod host), mammalian host immunity to closely related viruses, broadened geographic range of vectors, increased global travel, population growth and increased urbanization, or other environmental factors. It is likely that some combination of these factors has allowed for increased transmission of arboviruses like ZIKV.

The explosive outbreaks of ZIKV in Yap and French Polynesia that occurred several years before the current pandemic should serve as a reminder that epidemics occurring anywhere have the capacity to spread globally at an alarmingly rapid pace. We also have a limited understanding of how multiple species of arboviruses circulating in the same area and spread by the same vectors (ZIKV, DENV, CHIKV and YFV) may interact in the mosquito hosts, the mammalian hosts, or both, to enhance or inhibit their transmission or the disease they

cause. Recent work has shown that multiple arboviruses can be transmitted simultaneously without affecting vector competence [77, 78]. The effect co-infection has on virus emergence should be explored further. Finally, methods to control vectors or the ability to transmit pathogens must be followed through, such as traditional vector control methods involving insecticides and breeding site removal [79], as well as newer methods such use of *Wolbachia* [80, 81], genetically modified mosquitoes [82], and lethal mosquito traps [83], among others. It is unclear how effective these control measures will be, but action is needed in order to prevent the further spread of ZIKV as well as additional arboviruses.

Sexual transmission of Zika virus

History

From the discovery of ZIKV in Uganda in 1947 [15] until 2007, sporadic virological and serological evidence of non-epidemic ZIKV transmission among humans in endemic areas of Africa and Asia were reported [84–86]. The first documented ZIKV epidemic occurred in 2007 on Yap Island, in the Federated States of Micronesia [2, 87, 88], and another likely occurred in Libreville, Gabon the same year [10]. In all of these reports, mosquito bites were assumed to be the mode of virus transmission and spread. The first evidence suggesting sexual ZIKV transmission may occur was a self-case report by one of the authors of Zika virus disease after mosquito-borne infection in Senegal in 2008, and subsequent infection of the author's wife following his return to Colorado, USA [4]. While virus was never recovered, serological evidence suggested direct virus transmission from husband to wife after condomless vaginal intercourse occurred upon his return home but prior to his Zika virus disease symptoms, which included hematospermia and prostatitis. This initial evidence of ZIKV sexual transmission and urogenital pathology was eventually supported in a second case report from the Tahiti ZIKV outbreak in 2013, where a patient presented with recurring Zika virus disease symptoms, including hematospermia which was recorded two weeks after his last clinical episode [5]. ZIKV RNA and viral particles were recovered from the patient's semen and urine, but never from his blood. It was not until the spread of ZIKV to the Americas that multiple lines of evidence were obtained verifying sexual ZIKV transmission. A number of different case reports were published in the first four months of 2016 of travelers who were infected by mosquitoes in areas with known epidemic transmission, and then transmitted the virus to their sexual partners after they returned to countries where mosquito-borne ZIKV transmission was absent at that time [89–92]. The report by D'Ortenzio et al [90] was the first to isolate ZIKV viruses from both the index patient (from his semen) and his sexual partner (from her saliva) and match the genome sequences, which diverged by only four synonymous mutations. Following these have been numerous other reports, nearly all from traveler-associated cases [93]. To be clear, the speed and seasonality of ZIKV spread in the current pandemic strongly suggests that in non-sylvatic transmission cycles, mosquito-infectious viremia titers are readily reached in certain infected persons, and that this is the primary driver of ZIKV transmission. However, many questions remain regarding the nature, epidemiology and significance of sexual ZIKV transmission, particularly in areas where mosquito-borne transmission is prevalent.

ZIKV as a STI

Sexual ZIKV transmission is defined as transmission through vaginal, anal, and oral sex, including the sharing of sex toys [94]. The former two routes of transmission have been documented in many case reports. Transmission through oral sex has so far only been suggested as a possibility that cannot be ruled out, given that oral sex has been reported as having happened along with vaginal or anal sex in some case reports [90, 95]. We are not aware of documented ZIKV transmission from sharing of sex toys alone. Most reports providing evidence of sexual ZIKV transmission have documented an infected male as being the index case, but female-to-male transmission has also been reported [96]. Based on the evidence gathered from a multitude of studies, the World Health Organization and the Centers for Disease Control and Prevention in the United States have released recommendations to prevent sexual transmission of ZIKV [97, 98]. It should be noted that sexual transmission may be confounded in some rare cases by the possibility of direct, non-sexual transmission, which has been described from a moribund patient who had a very high level of viremia and eventually died, but apparently transmitted ZIKV to one of his family members who was serving as one of his caregivers [99].

Presence and persistence of ZIKV in body fluids of infected patients

ZIKV RNA has been detected in blood samples (serum, plasma and whole blood), male and female reproductive tract samples, urine, saliva, breast milk, conjunctival and other body fluids of infected humans [100]. Infectious virus has been recovered from most of these body fluids as well, but more rarely [101]. Recent reviews have detailed ZIKV kinetics and the range of titers in these different biological samples from the many published case reports [93, 102, 103] and from the prospective ZiPer study [6] which calculated the median time until ZIKV RNA clearance from the serum of 132 infected patients as 13.9 days [11.2–16.6]. While ZIKV RNA has now been reported to persist for up to 2–4 months in whole blood [104–106], infectious particles were not successfully cultured from these late-phase blood samples. Given the need for a threshold blood titer to infect mosquitoes [107], the ability of such late-phase samples to infect mosquitoes could be questioned. It is also not clear from these reports that viral kinetics or titer ranges in blood or saliva differs between males and females, and it remains to be seen if the route of infection (mosquito bite or sexual transmission) might influence these factors.

Presence and persistence of ZIKV in the male urogenital tract

In infected men, ZIKV RNA has now been detected in semen (defined as ejaculate or seminal fluid that may or may not contain spermatozoa) in more than a dozen case reports [5, 90, 95, 104, 108–117], and in over half of the infected, enrolled men in the ZiPer prospective study in Puerto Rico [6]. Many reports have documented high viral RNA copy number in semen relative to other infected body fluids [5, 90, 114], while other reports have not seen evidence of this [110]. The ZiPer study calculated the median time until ZIKV RNA clearance post onset of symptoms from infected patient urine and semen as 8.2 days [6.4–10.0] and 34.4 days [27.9–40.8], respectively; importantly, the estimate for urine was made from data combining both male and female patients. The detection of viral RNA in semen and urine of men has been reported for up to 188 and 91 days post onset of

symptoms, respectively [115], and infectious virus has been isolated from semen more than two months after symptoms [108]. In total, these data indicate ZIKV tropism for tissues of the male urogenital tract and the potential for long-term shedding from urogenital tract fluids. Indeed, male-to-female sexual transmission has occurred more than 30 days post illness onset in an index case [118], and indications of urogenital pathology in several of the case reports from males, including hematuria [86], hematospermia and prostatitis [4], hematospermia [5], and microhematospermia [119] provides further evidence of this tropism. The exact cells or tissues infected by ZIKV in the male reproductive tract remains unclear, but several studies are beginning to answer this question. One or more tissues of the accessory genital glands (the seminal vesicles, the prostate gland and the bulbourethral glands) are likely infected given the several reports of hematospermia listed above, and which is often unnoticed and most commonly associated with trauma or infection of the prostate [120]. Importantly, ZIKV has been detected in the ejaculate of at least 2 vasectomized men [104, 108] and one man who had non-obstructive azoospermia [121], and sexual ZIKV transmission has occurred from two of these cases [108, 121]. We also can verify that one of the authors of this paper (who is the male patient from the first sexual transmission report in Colorado) had a vasectomy in 2007, approximately 1 year prior to the sexual transmission event; this information was not described in that initial report. However, this evidence does not preclude the possibility that cells and tissues of the testes are infected with ZIKV. In one publication, virus antigen was detected by immunohistochemistry in the head of spermatozoa obtained from an infected individual [122].

Presence and persistence of ZIKV in the female reproductive tract

As with men, ZIKV infections of female travelers to endemic regions, which were not linked to sexual infection, can be detected by the presence of viral RNA or virus in urine and vaginal swabs, suggesting urogenital tract tropism following infection from a mosquito bite occurs [123]. Persistence of ZIKV RNA in female urogenital fluids (genital and endocervical swabs or cervical mucus) has been recorded up to day 14 after symptoms onset [124] [125]. As would be expected, ZIKV RNA or virus can also be found in female urogenital fluids and/or tissues following documented sexual transmission events [90, 96, 108, 111, 113, 118, 126]. While seemingly likely, it is not clear yet whether sexual transmission more often leads to ZIKV urogenital infection of females relative to transmission from a mosquito bite. In the ZiPer prospective study [6], ZIKV RNA was detected in the vaginal secretions of only 1 of 50 women participants who provided vaginal swab samples, but these women were not stratified as to their suspected route of infection. Conversely, ZIKV RNA was detected in genital and endocervical swabs and cervical mucus from follow-up examinations of 5 out of 5 women who were enrolled in fertility program in Guadeloupe and found to have been infected with ZIKV [127]. Again, the suspected infection route was not determined. A finer study of infected tissues of the female urogenital tract in humans is necessary to determine which tissues are preferentially infected, and if differences in tissue tropism or viremia occur when infected by mosquito bite or sex.

Animal models of ZIKV sexual transmission and infection

Animals models of both male and female ZIKV infections of the urogenital tract and sexual ZIKV transmission will help understand tissue tropism and viral spread, but their relevance

to human infection and disease must be critically evaluated. Immunocompetent mice are generally resistant to ZIKV and so to elicit infections, researchers typically must use immunocompromised mice strains (e.g. interferon receptor knockout strains) or immunocompromising experimental procedures, such as administering monoclonal antibodies against interferon receptors [128], or treating mice with immunosuppressing drugs [129].

Wild type male mice treated with antibody against the interferon receptor have testicular pathology when infected with ZIKV, and high levels of virus and ZIKV RNA in the testes (including Sertoli cells and spermatocytes), epididymis and epididymal fluid [130]. In *Ifnar* $I^{-/-}$ mice, ZIKV can also be produced in high titers in the testes [131] and it infects the Leydig cells, leading to a decrease in testosterone production, testicular atrophy and hypofertility [132]. AG129 mice (mice lacking both the type I and type II interferon receptors) ultimately succumb to encephalitis when infected with ZIKV. In this model, testicular inflammation was apparent and immunostaining of ZIKV antigens occurred in the epididymides and testes of ZIKV-inoculated mice [11]. Frequent sexual ZIKV transmission was seen from these mice by examining the ejaculate deposited in the uteri of mated females, which could contain $>5 \log_{10}$ PFU. Furthermore, vasectomized and then infected males could still transfer virus in their ejaculate to the females. Immunocompromised male hamsters (knockouts of the STAT2 gene) are also susceptible to ZIKV infection with infectious viral particles being mostly found in the testes of infected animals when compared to the spinal cord, brain and kidney [133].

AG129 female mice that become pregnant when mated to infected AG129 males had higher mean ZIKV titers in their uteri, and placental and fetal infections occurred in some of the infected pregnant females [11]. Wild type female mice can be transiently infected in the lower reproductive tract following direct intravaginal inoculation of ZIKV and after the mice are induced into diestrus phase by an injection with medroxyprogesterone (Depo-Provera) [134, 135]. In immunocompromised hamsters, fetal infection occurs after pregnant females are infected subcutaneously [133].

Likely more relevant to humans, immunocompetent non-human primates infected with ZIKV produces viremias, virurias and clinical signs that resemble those in humans [136–140]. In one study, ZIKV RNA was detected in testes, but not in the prostate or epididymes, of two rhesus monkeys infected subcutaneously with the virus [138]. In the study by Hirsch et. al [137], ZIKV RNA could be found in the infected rhesus female vagina and uterus and the rhesus male prostate, seminal vesicles, urine and urethra, but not the testes. Osuna et. al [140] detected high viral loads and persistent shedding of ZIKV in the semen up to 3 weeks after viremias resolved, but only sporadic and a low level of ZIKV RNA shedding was detected from female vaginal swabs.

In the latest study, 50% and 75–100% of rhesus and cynomolgus macaques become viremic after intravaginal and intrarectal infections, respectively, and all seroconverted [141]. Importantly, viremia level in some of these animals was 10–100 fold higher than that expected to infect mosquito vectors. In their report, the authors mention that sexual transmission may help maintain an enzootic cycle among NHPs. It is notable that none of

the animal model studies reported to date have infected the animals by mosquito bite, which may influence virus tropism or disease presentations.

Mosquito ZIKV transmission compared to sexual ZIKV transmission

In future studies, it will be very important to determine the potential differences in factors that influence relative transmission success and disease presentation between sexual and mosquito ZIKV transmission co-occurring in ZIKV endemic areas. These factors may include virus infection thresholds, disease risk, prevalence, incidence and outcome (especially between females and males), and viral tissue tropism and persistence in body fluids or tissues. Studying these potential differences is inherently confounded by the fact that adult *Ae. aegypti* rarely travels more than 30 m per day [142], suggesting that arboviruses in these vectors are more often transmitted within the area of a typical household. Indeed, epidemic DENV transmission in Peru has been linked to person movement between households [143]. Assuming sex between couples happens in the same households where ZIKV-infected vectors may be biting both partners, detailed pathological, serological and/or virological studies may be the only way to sort out infections arising from either route. For example, studies of ZIKV genetic sequences taken from urogenital tissues or suspected sexual transmission events [144] may uncover unique signatures compared to those from infected blood that are known to be from mosquito bite transmission.

Perhaps the most important question related to these issues is whether sexual ZIKV transmission, more often than mosquito transmission, leads to placental or fetal infection in pregnant women and may be more often linked with spontaneous abortions or Zika virus congenital syndrome. This largely remains unanswered at present, but no doubt is being actively pursued in the many epidemiological studies of pregnant women and those attempting to become pregnant and who reside in areas of active ZIKV transmission. The quantity of ZIKV transmitted between the mosquito and sexual routes, and locations of virus deposition from each, are primary factors driving this question. If male-to-female sexual ZIKV transmission occurs during pregnancy, more than 10^8 PFU of virus [90, 114], given an ejaculate volume of ~3.7 mL [145], can be deposited in very close proximity to the developing placenta and fetus and possibly be able to directly infect these tissues. Conversely, infectious mosquitoes inject saliva containing virus into skin (often on the extremities) when probing and blood feeding, most of which is deposited extravascularly ($\sim 10^3$ – 10^6 PFU) and little of which is injected intravascularly ($\sim 10^2$ PFU) [146]. Considering epidemiology, there is a relatively high prevalence of sexually transmitted infections among pregnant women, especially those from developing countries, including trichomoniasis, bacterial vaginosis and HIV [147, 148]. Spontaneous abortions and Zika congenital syndrome have been reported to occur more often when infection occurs in the first trimester [149]. While epidemiological studies have shown that the frequency of coitus tends to decline over the course of pregnancy [150–152], pregnant women, especially those in developing countries, more often report having sex without a condom compared to non-pregnant women [151, 153].

Another very important question is whether sexual transmission leads to different ZIKV disease symptoms, prevalences and/or incidences in female patients. As far back as the Yap

Island outbreak, where an estimated 73% of the island's population was infected, it was observed that 61% of the cases were female and the sex-specific disease attack rate was higher in females than males (17.9 per 1000 females vs. 11.4 per 1000 males), despite the evidence that males were more likely to have anti-ZIKV IgM than females (77% vs. 68%; relative risk = 1.1 [1.0–1.2]). In the US, the percentage of women among travel-associated ZIKV-infections (including ones involving sexual transmission) from January 2015 to February 2016 was 65% (75/115) [154], and a study in Rio de Janeiro found an 88% higher age-adjusted ZIKV incidence in women versus men within sexually active age groups and suggested the results may be due to sexual transmission [155]. This study also used dengue infections as a comparison, in which only a 30% higher age-adjusted DENV incidence in women was observed. In the largest study, Dos Santos et al. [156] examined more than 150,000 ZIKV disease cases across seven countries and observed that females had a 75% higher incidence rate over men (rate ratio = 1.74 [1.71–1.79]). It is important to note that these data may be confounded by differences in targeted enrollment procedures or differential behaviors exhibited by women compared to men, such as those that may influence mosquito-bite risk or health-care seeking frequency. Future studies should be controlled for these confounders and compared with ZIKV exposure measures, as was presented in the Yap Island study. For example, a large study in Puerto Rico found that 61% of females among the 29,345 ZIKV qRT-PCR cases [157] were non-pregnant women, which may at least remove the effect of frequent health-care seeking behavior among women due to pregnancy. Lastly, a prospective cohort of acute febrile cases who attended a health unit in the Recife Metropolitan Region, Brazil showed a female bias in patients infected with ZIKV, as opposed to CHIKV-infected patients and those not infected with ZIKV, CHIKV or DENV (Magalhaes T & Marques ETA, unpublished). Importantly, in this study gender distribution among all patients enrolled did not differ, and CHIKV-infected patients actually exhibited a male bias.

Similar to the above question of whether sexual transmission may more often lead to fetal infections, it is also important to understand whether infection by the different transmission routes might affect disease presentation or severity, such as examining whether certain neurological symptoms or long term sequela more often appear from known sexual transmission events. We are currently also lacking human data on whether sexual ZIKV transmission might be more prevalent with the Asian compared to the African lineage virus, however the first sexual transmission case described was from an infection occurring in Senegal, and so presumably was due to an African lineage virus. Regardless, knowledge of epidemiology of sexual transmission in endemic areas of Africa is non-existent and studies on this should be undertaken.

Lastly, while several important issues need to be addressed for each type of transmission, it is important to keep in mind that these modes of transmission may synergize to extend the ZIKV transmission cycle. For instance, the data from intravaginal and intrarectal inoculation of NHPs shows a delayed viremia [141], which suggests that, if this is also observed in humans, sexual transmission in the same household where mosquito biting is occurring might extend the time of effective mosquito infections from viremic individuals in that household. Although not yet demonstrated, there may also be differences in viremia titers

between persons infected sexually compared to those infected by mosquitoes, which would potentially increase the persons in a household who would be infectious to mosquitoes.

Acknowledgments

This publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award numbers R01AI067380, R21AI125996 and R21AI129464. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Highlights

- Zika virus (ZIKV) is unusual among the mosquito-borne flaviviruses in that it is transmitted by mosquitoes and through sex, but much remains to be discovered about the relative influences of these two transmission routes in ZIKV ecology, infection and disease.
- Mosquito-borne ZIKV transmission in both sylvatic and urban cycles seems to be mostly from *Aedes* species, and this is the likely driver of the rapid and widespread nature of the current pandemic.
- Sexual ZIKV transmission is driven by a urogenital tissue tropism of the virus in males that can result in high and persistent viral titers in semen, and may be causing more frequent disease and severe pathology among women compared to men.