ZIKA-How fast does this virus mutate?

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ABSTRACT

The World Health Organization has declared the present Zika virus epidemic to be a 'Public Health Emergency of International Concern'. The virus appears to have spread from Thailand to French Polynesia in 2013, and has since infected over a million people in the countries of South and Central America. In most cases the infection is mild and transient, but the virus does appear to be strongly neurotropic and the presumptive cause of both birth defects in fetuses and Guillain-Barré syndrome in some adults. In this paper, the techniques and utilities developed in the study of mitochondrial DNA were applied to the Zika virus. As a result, it is possible to show in a simple manner how a phylogenetic tree may be constructed and how the mutation rate of the virus can be measured. The study showed the mutation rate to vary between 12 and 25 bases a year, in a viral genome of 10 272 bases. This rapid mutation rate will enable the geographic spread of the epidemic to be monitored easily and may also prove useful in assisting the identification of preventative measures that are working, and those that are not.

Keywords: Zika; Virus; Polyprotein; Mutation rate; Phylogenetic tree

INTRODUCTION

On 01 February, 2016, the World Health Organization declared the emerging Zika epidemic to be a 'Public Health Emergency of International Concern' (PHEIC), highlighting that this epidemic is now considered to be a major threat to the whole world (WHO, 2016). Their statement of intent includes the lines: Appropriate research and development efforts should be intensified for Zika virus vaccines, therapeutics and diagnostics; and, National authorities should ensure the rapid and timely reporting and sharing of information of public health importance relevant to this PHEIC.

As a consequence, it can be expected that many research institutions will increase their studies into Zika and related viruses and many new scientific papers will appear in the coming months. At the same time, it is expected that many more RNA sequences of the virus will appear in the public domain.

Also, as a result of the rapidly increasing importance of the Zika virus, it is likely that scientists and physicians who normally would not study the genetics of a virus might start examining the newly available data.

The Zika virus is a *Flavivirus* carried by mosquitoes and was originally found in a Rhesus monkey placed in the Zika forest of Uganda in 1947, as described by Haddow et al. (1964). Over the last 60 years it has been the cause of epidemics in several African countries. However, in 2010 the virus spread to parts of Asia, in particular to Thailand (Fonseca et al., 2014; Haddow et al., 2012), and by 2013 had reached French Polynesia (Baronti et al., 2014). Since then there has been an explosive epidemic affecting the populations of many countries in both South and Central America. At the present time this epidemic shows no signs of abating.

In the many small epidemics that have occurred, there has been no indication of the virus causing anything but mild and transient infections. In the recent epidemic in Polynesia, however, cases of central nervous system damage have been observed and described as being a form of Guillain-Barré disease (Korff, 2013; Winer, 2014). In the current Brazilian epidemic, the emphasis has been on the possibility of an association with birth defects, especially microcephaly, resulting from maternal infection with Zika in the first and second trimester of pregnancy (Mlakar et al., 2016). The presumptive link between Zika infection and microcephaly is now looking more and more likely. Further cases of Guillain-Barré syndrome have also been seen.

The Zika virus is closely related to the viruses of Yellow, Dengue and West Nile fever, all of which cause significant illness and mortality. However, there are many other flaviviruses that are less well known and their hosts include horses, sheep, bats, birds and many other animals. A paper in 1998 listed over 70 different flaviviruses (Kuno et al., 1998), with new ones continuing to be identified (Moureau et al., 2015).

All flaviviruses appear to have much the same structure. The mature virus particles, virions, are about 50 nm in diameter and icosahedral in shape. Modern electron microscopy can show

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virions in considerable detail (Zhang et al., 2013; Zhou, 2014). The outer part is formed by an envelope overlying a phospholipid bi-layer membrane and the core contains a single stranded RNA molecule of about 10k bases.

In the mature Zika virion, the RNA molecule, which encodes a polyprotein, is described as having 10 272 bases, or 3 424 3-base codons for specific amino acids. The translation of bases to functional codons is not perfect, but for analysis purposes it has become accepted to describe the structure of the molecule in this manner:

starting with MKN … and ending with … GVL

(i.e. The codons for: methionine, lysine, asparagine …….

…… glycine, valine, leucine).

The polyprotein is a linear assembly of both structural and non-structural genes. The structural genes are for the envelope, membrane and capsid, and the non-structural genes are usually *NS1*, *NS2A*, *NS2B*, *NS3*, *NS4A*, *NS4B*, and *NS5* (Bollati et al., 2010). For the purposes of this paper, this simple explanation will suffice.

The envelope and membrane genes define how the outer part of a virion is conformed. This outer part is important as it acts as an antigen for antigen-antibody reactions and also in the interaction between the virus and entry receptors when a virion attempts to enter a cell. Consequently, mutations affecting the construction of the envelope and membrane are probably more significant than mutations in other parts of the genome, and perhaps of greater influence when it comes to possible changes in virulence.

It remains unclear as to which cells, if not all cells, in humans are susceptible to invasion with the Zika virus. However, the cells of the central nervous system do appear to be especially vulnerable (Mlakar et al., 2016). In all instances, the process appears to be the same, whereby a virion attaches itself to the entry receptors on the outer surface of the cell and then enters the cell in a process described as endocytosis (Perera-Lecoin et al., 2014).

Once inside a human cell, the envelope and membrane separate from the core. The virus then hi-jacks the cellular apparatus for its own purposes. The polyprotein is copied and cleaved into its constituent parts and daughter virions are produced, each containing its own copy of the polyprotein (Clark & Harris, 2006).

At present, there are few drugs that prevent replication of the Zika virus, and the older and well-established antiviral drugs, such as amantadine, which are active against the influenza virus, are not helpful against flaviviruses (Oxford et al., 1970). However, a great deal of work is being done to find new antiviral drugs (Sampath & Padmanabhan, 2009). It is interesting to note that a traditional Chinese remedy, Xiyanping, was used for its anti-viral properties in the treatment of a recent case of Zika (Deng et al., 2016).

In relation to the present epidemic, the ability of the Zika virus to enter cells of the placenta and the central nervous system is particularly important. For now, however, it is unclear whether or not these invasions are more dependent on the strain of the virus, the genetic make-up of the host, or some other factors. Once the virus has crossed the placental barrier to enter the fetus or the blood-brain barrier to enter the central nervous system, it is likely that the usual antigen-antibody reactions are lessened and the virus is able to proliferate more easily. It is also unknown as to how long it might take for the virus to be cleared from the fetus or central nervous system, although it does seem likely that virus replication can continue in these areas for many months.

MATERAILS AND METHODS

GenBank database

The RNA sequences for the Zika virus in the public domain can be found in the GenBank database of the National Institute of Health (Benson et al., 2013). At present (March 2016), there are 16 complete sequences from viruses collected in Africa and Asia before the start of the present epidemic and 17 sequences produced since. Details of these sequences are given in Table 1.

The corresponding page on the GenBank database for a given sequence can be found by using a URL of the form: http://www.ncbi.nlm.nih.gov/nuccore/KU744693. Each page gives the amino acid list and nucleotide base FASTA file for the RNA sequence. However, a GenBank page contains no real explanation as to what each list or file might mean and for this reason the author has developed a pair of Zika virus utilities that allow the user to compare one sequence with another.

Zika virus utilities

In conjunction with this paper, two simple utilities were prepared and can be found on the author's website in the form of two webpages (www.ianlogan.co.uk/zikapages/zika.htm). From there the user can choose either the Amino Acid Analyser or the Nucleotide Base Analyser

The Amino Acid Analyser has in its source file copies of the amino acid lists for all complete RNA sequences found in the GenBank database and a small JavaScript program that allows the user to compare any sequence against any other. The results are displayed as a list of amino acid changes.

The Nucleotide Base Analyser has in its source file copies of the FASTA files for the complete RNA sequences of all sequences in the current epidemic. Again, a small JavaScript program enables the user to compare two sequences and show the mutational differences between them.

Although the webpages can be viewed with any commonly used web browser, the author recommends MOZILLA FIREFOX as it allows the user to alter the size of the text area, if needed.

It is the author's intention to keep these webpages up-to-date as new Zika RNA sequences appear on the GenBank database.

RESULTS

Non-synonymous amino acid changes observed in the present epidemic

A mutation that causes a non-synonymous change of an amino acid is often considered to be significant. However, if the change is between amino acids of similar size and polarity, there is probably no effective change in the functioning of the target protein.

Table 1 ZIKA RNA sequences in GenBank database (01 March 2016)

Accession no.	Country of origin	Date of collection
African sequences		
1 LC002520	Uganda	1947
2 HQ234499	Malaysia	1966
3 HQ234500	Nigeria	1968
4 KF383116	Senegal	1968
5 KF383115	CAR	1968
6 HQ234501	Senegal	1984
7 KF268948	CAR	1976
8 KF268949	CAR	1980
9 KF268950	CAR	1980
10 KF383117	Senegal	1997
11 KF383118	Senegal	2001
12 KF383119	Senegal	2001
Asian sequences		
13 EU545988	Micronesia	2007
14 JN860885	Cambodia	2010
15 KU681082	Philippines	2012
16 KU681081	Thailand	2014
Current epidemic		
Brazilian reference sequence		
17 KJ776791	Polynesia	2013
18 KU365779	Brazil	2015
19 KU365778	Brazil	2015
20 KU365777	Brazil	2015
21 KU365780	Brazil	2015
22 KU312312	Suriname	2015
23 KU501215	Puerto Rico	2015
24 KU509998	Haiti	2014
25 KU321639	Brazil	2015
26 KU527068	Brazil	2015
27 KU647676	Martinique	2015
28 KU501216	Guatemala	2015
29 KU501217	Guatemala	2015
30 KU707826	Brazil	2015
31 KU497555	Brazil	2015
32 KU740184	China	2016
33 KU744693	Venezuela	2016

The amino acid changes shown by the Zika RNA sequences in the present epidemic are listed in Table 2. The table demonstrates that by using this method the sequences can be split into 12 different strains with between 0 and 25 amino acid changes.

The mutation M2634V is common to all virus strains that have come from countries in South and Central America and is caused by the base mutation A7900G. However, as this mutation is found in the NS5 gene, it is unlikely to be of significance as to the virulence or general behavior of the Zika virus. The NS5 gene is involved in the replication of new virions and is not a structural gene (Zhao et al., 2015). It is perhaps too early to say that this mutation has absolutely no effect, but for the moment the M2634V mutation can be seen as a useful marker to the present epidemic.

Base mutations in samples collected in the present epidemic

While there are relatively few non-synonymous mutations in the virus strains collected in the present epidemic, there are many more synonymous mutations (i.e., mutations that do not produce a change of amino acid),and as a result a phylogenetic tree can be constructed.

Figure 1 shows the phylogenetic tree produced by using the mutations from the 17 complete Zika sequences currently found in the GenBank database. This figure shows the virus samples can be separated into 15 different strains, considering the sequence pairs KU365777/KU365780 and KU365799/KU707286 as being from two strains.

Estimation of the Zika virus mutation rate

The data presented in Figure 1 show that actual mutations vary between 9 and 64 for the sequences collected during the present epidemic. This number was calculated by considering the mutations that have occurred since the outbreak of the epidemic in Brazil, and Figure 1 suggests the use of a hypothetical Brazilian Reference Sequence (BRS) to describe a possible sequence for the original strain arriving in Brazil.

The number of mutations found in a sequence appears to be proportional to the date on which the original sample was collected. The early sequences show between 9 and 30 mutations, whereas the two latest sequences, KU740184 and KU744693, show 30 and 64 mutations, respectively. The latter sequence is from a sample collected on 6 February, 2016, and shows that the Zika virus continues to mutate at a rapid rate.

As the present epidemic can be considered to have started in Polynesia in 2013 (Baronti et al., 2014) and has now lasted about 2.5 years (i.e., late 2013 to early 2016), the mutation rate appears to vary between about 12 to 25 mutations a year. The genome of the Zika virus is normally considered to be a polyprotein of 10 272 nucleotide bases, so the mutation rate can also be considered as changing between 0.12% and 0.25% of the RNA polyprotein each year.

It is not possible, using the data presently available, to provide a more accurate value for the mutation rate. However, the suggested rate of 12 to 25 mutations a year would appear to be a suitable starting point for further studies.

DISCUSSION

Present epidemic

The decision by the World Health Organization to declare a Public Health Emergency in February 2016 due to the threat of

Table 2 Non-synonymous amino acid changes found in sequences from the present Zika virus epidemic

GenBank accession no. -Country of origin-Date of collection	Amino acid changes					
KJ776791-Polynesia-2013						
KU365778-Brazil-2015	M2634V					
KU365779-Brazil-2015	M2634V					
KU707826-Brazil-2015	M2634V					
KU365777-Brazil-2015	M2634V	N2778D				
KU365780-Brazil-2015	M2634V	N2778D				
KU312312-Suriname-2015	M166T	T769A	M2634V			
KU501215-Puerto Rico-2015	I80T	A2611V	M2634V			
KU527068-Brazil-2015	K940E	T1027A	M1143V	T2509I	M2634V	
KU647676-Martinique-2015	D107E	R1118W	I1226T	M2634V	T3353A	
KU501216-Guatemala-2015	V346I	G894A	M2074L	M2634V	K2694R	R3045C
KU501217-Guatemala-2015	V346I	G894A	M2074L	M2634V	K2694R	R3045C
KU497555-Brazil-2015	S550T	L1259F	M2634V	E2831V		
KU740184-China-2016	D107E	D445G	I1285V	M2634V	T2749I	V2787A
KU509998-Haiti-2014	Y916H	H1857Y	I2295M	I2445M	M2634V	
KU321639-Brazil-2015	Y916H	H1857Y	I2295M	I2445M	M2634V	
KU744693-Venezuela-2016	E76D	V323A	I442L	V503A	D520A	L612V
	H613D	V620G	A623G	F739I	A794G	D795G
	S970W	R1005W	T1050A	C1107S	R1118Q	D1856E
	H1857Y	S1867R	D1938G	I2295M	A2313P	T2317S
	D2419E	I2445M	M2634V	S2807A	H2809K	E2831D
	P2833A	N2974I	M2975T	V3334A		

The change M2634V (Methionine to Valine), common to all sequences, occurs in the *NS5* gene and is the result of the base mutation: A7900G, which changes the codon from 'ATG' to 'GTG'.

a Zika virus pandemic may be thought a pessimistic move. However, the evidence appears to indicate that the Zika virus is no longer restricted to localized habitats nor largely dependent on the monkey as its host. It now covers a much larger area in South and Central America where it is wholly dependent on the human as its host. Unfortunately, there appears to be little, if any, herd immunity against the virus in the populations of these countries, despite the closely related Dengue virus being prevalent.

The sudden spread of Zika to South and Central America does not appear to have been due to any change in the mosquito vector or anything known to make the Zika virus more virulent. Rather, it appears related to the fact that infected people are now able to fly rapidly from country to country, thereby spreading the disease very easily. This means there is little to stop the epidemic continuing to spread to other populations that also have low levels of herd immunity against the virus.

The absence of mosquitoes and the low incidence of person-to-person spread of the virus will probably mean the epidemic will not spread in countries of the southern and northern latitudes. From evidence obtained so far, however, it would appear likely the epidemic is only at its earliest stage and any suggestions as to what might happen remain speculative (Bewick et al., 2016).

Zika phylogenetic tree

The two utilities prepared for this study show that many distinct strains of the Zika virus now exist, even though the present epidemic is less than three years old. When considering just the non-synonymous mutations in the RNA, it is possible to define 11 strains in the present epidemic. However, a more detailed examination looking at the actual mutations of the available sequences distinguishes 15 strains. As more data are made available, it is expected that the number of identifiable strains will increase. The phylogenetic tree shown in Figure 1 suggests the beginning of a geographical spread of the associated virus strains, with distinct strains now coming from Martinique, Guatemala, Puerto Rico and Suriname, whilst Brazil continues to show a mix of strains.

Estimation of the Zika mutation rate

The data used in building the phylogenetic tree can also be used to estimate the mutation rate of the Zika virus, which appears to vary between about 12 to 25 mutations per year, equivalent to 0.12%-0.25% of the RNA mutating each year. This rate is very high when compared to the human DNA mutation rate, where a period of perhaps 250 000 years might be expected (Logan, 2015). Nonetheless, it is not really appropriate to compare RNA mutations against DNA chromosomal mutations as DNA replication is a self-correcting process, whereas RNA duplication is liable to many sorts of errors.

However, the clear evidence of a high mutation rate in the Zika virus will allow for the present epidemic to be tracked in a fairly simple manner, and should be helpful in identifying where local mosquito prevention initiatives are working and where they are not.

Zika infection complications

A particular feature of the present epidemic is the presumptive link to the high incidence of fetal abnormalities and cases of Guillain-Barré syndrome. These two complications appear to be

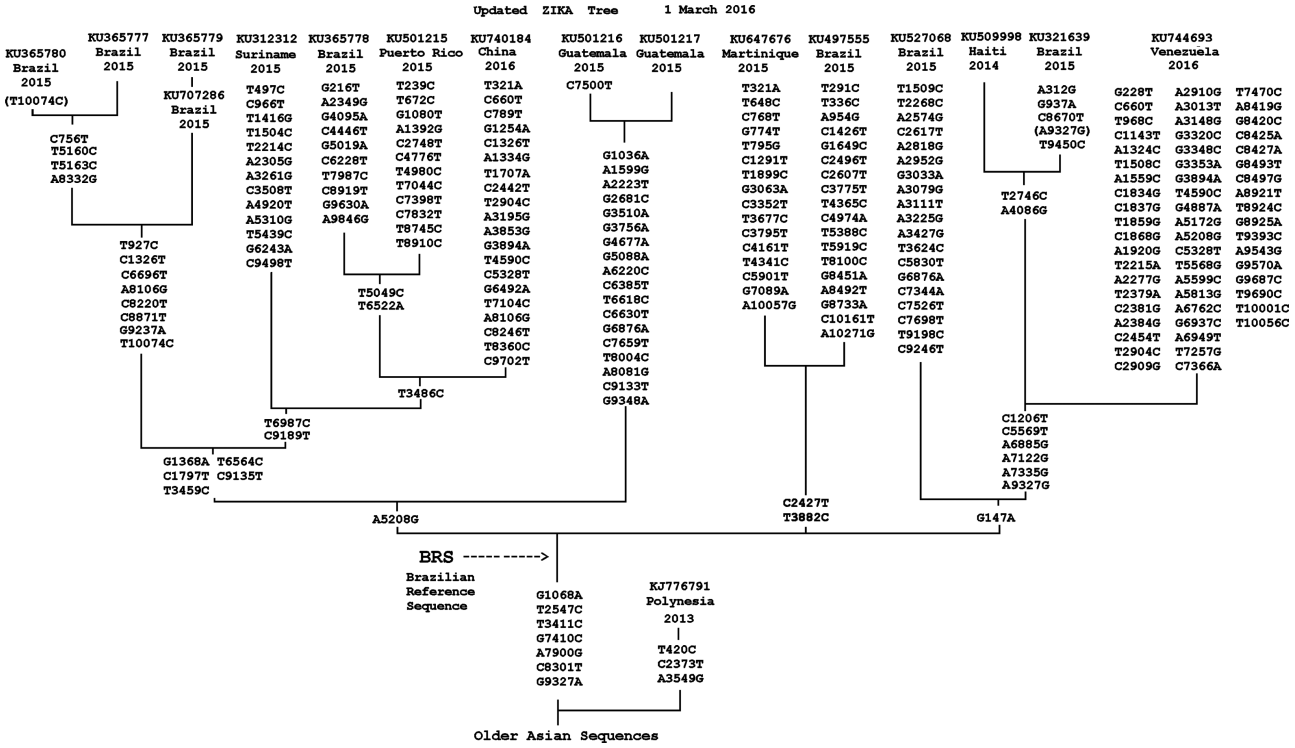

Figure 1 Phylogenetic tree of the 17 Zika RNA sequences from samples collected in the present epidemic

Two missing mutations - indicated by the brackets: C10074T in sequence KU365780-Brazil-2015 and G9327A in sequence KU321639-Brazil-2015, were probably caused by technical errors. The position of a hypothetical Brazilian Reference Sequence (BRS) is marked on the tree. The BRS is used in the two utilities, the Amino Acid Analyser and Nucleotide Base Analyser prepared for this paper and available at: www.ianlogan.co.uk/ zika-pages/zika.htm.

caused in very different ways, with fetal abnormalities possibly being the result of direct fetal infection, and Guillain-Barré syndrome cases possibly due to an exaggerated auto-immune response (Cao-Larmeau et al., 2016; Willison et al., 2016).

In the author's opinion, however, both conditions may result from the same underlying cause, in which the virus is able to cross the normally impenetrable placental and blood-brain barriers. How this happens is unclear, but it might just be a matter of a person getting a very high initial infection, possibly by having been bitten by a physically large carrier mosquito, or being bitten by several carrier mosquitoes in a very short period of time.

A study using the West Nile virus (Styer et al., 2007) showed that whilst most of the inoculum from a mosquito bite remains localized in the skin, there is always a significant initial viraemia. In this respect, a recent report from Slovenia (Mlakar et al., 2016) showed the X-rays of an affected fetus having numerous calcifications in the placenta and brain. Whilst it is unproven, it would seem possible that these lesions result from localized 'viral plaque formation' associated with an initial viraemic spread. A similar clinical picture is seen in tuberculosis. Although this disease is caused by a bacterium and not a virus, the resulting X-ray picture of localized calcifications is well-recognized and is termed miliary tuberculosis (Khan et al., 2011; Yang et al., 2015).

It is also possible that the risk of developing complications from the Zika virus may reflect the genetic differences between sufferers and the general population. At the present stage of our knowledge, however, there is no indication of what particular differences might be important.

CONCLUSIONS

This study shows in a simple way how sequencing data from samples of the Zika virus available in the public domain can be collected and analyzed. Using this data, it is possible to construct a phylogenetic tree and show that in the present epidemic there are already many identifiable virus strains. The data also show that the Zika virus has a high mutation rate.

This short paper raises as many questions as it tries to answer. The present epidemic is from the Zika virus, but Yellow Fever cases are rising in Africa and Dengue affects millions of people each year. Thus, further pandemics caused by flaviviruses, other than Zika, pose a continuing threat.

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