May Phylogenetic Analysis Support Epidemiological Investigation in Identifying the Source of HIV Infection?

the border between probability and certainty

Massimo Ciccozzi
(1957--?????? Untill 2052 I hope)

Vincent Willem van Gogh (1853 – 1890)
maybe
CLASSIC EPIDEMIOLOGY
- Surveillance
- Cross sectional studies
- Case – control studies
- Cohort studies

NEW EPIDEMIOLOGY
- Phylogenetic analysis
- Evolutionary analysis
- Phylodinamic analysis
- Geographical analysis

HUMAN POINT OF VIEW

MICRORGANISM POINT OF VIEW

THE BIG FEAR

Or

THE BIG COLLABORATION
ART Decreases Death due to HIV-1 Infection

Mortality among 16-44 year old Americans; 1987-2000
Among HIV-1 subtypes, clade A has been further classified into 4 subsubtypes (A1-A4).

HIV-1 A1 subtype predominate in the Russian federation and in other Eastern European countries as a monophyletic variant originated in Central Africa.

After an initial diffusion via heterosexual contacts, A1 variant spread explosively among IDUs and their partners.

Among non-B subtype HIV-1 A1 in Italy accounts for 12.5%.
The Bioinformatics paradigm in molecular evolution

Aligned DNA (or aa) sequences in a homologous gene region

Model of evolution

Tree construction algorithm

Evaluation of tree reliability

Using different tree-building algorithms
- Bootstrap analysis
- Maximum likelihood based-test

Formulating
Specific questions

Evolutionary patterns
- Coalescent models
- Phylogenetics

Experimental data, i.e.:
- HIV quasispecies from different patients
- HIV/HCV longitudinal samples
- Specific patient cohorts (slow/fast progressors, cancer patients, naïve/HAART patients, etc.)
The molecular typization of microorganism

The PCR application:
• Molecular epidemiology and phylogenetic analysis
• Drug resistance studies
• Virulence
• Mutants
Viral Molecular Evolution

Viruses normally lives in an environment (host) in continue evolution

Viruses must be always able to answer at change environmental conditions

• Immune protection
• Passage from a guest to the other
• Drugs

Virus must be ahead always a footstep to the guest

Evolution = variation + natural selection

Then a virus must Vary
Molecular evolution and virulence evolution

1) Microorganisms to low virulence can modify this own characteristic in consequence of changes in the state of immunity of the infected population.

2) Particularly the use of defective vaccines can conduct to an evolution toward one increased virulence of the microorganism (Gandon 2001; Mackinnon 2004).

Heath Ledger in “Batman the dark knight”
Molecular clocks: a controversial theory

"Imagined you that for years you are regulated you, to know the time, in base to the old clock in the plaza of the country...

One day you decide to know how this clock works. You climb you on the bell tower, you open the door of the room where you should find the mechanism of the clock and that that find it is only guano of pigeons, mice dead and other things unmentionable-nothing that can move the hands of the clock in regular way.

You would conclude that the clock cannot work at all"

Roger Lewin, New Scientist 1990.
phylogeny

• It has the purpose to Reconstruct the evolutionary history of the species

• to Esteem the time of divergence of two species

• to Tell the sequence of events along an evolutionary line

• That is to reconstruct the History passed of the organisms, studying and Comparing among them those actual (historical reconstruction on comparative base)

May Phylogenetic Analysis Support Epidemiological Investigation in Identifying the Source of HIV Infection?
We describe here a case of HIV-1 infection in a 24-year-old Italian soldier in service in Kabul, Afghanistan, where his military command was placed.

The epidemiological investigation

In September 2009, during a laboratory investigation in the Military Barracks based on demand screening, he was found to be positive for anti-HIV antibodies (by an ELISA test), which was confirmed by Western blot.

The viral load was 39,882 copies/ml and the CD4 cell count was 568 cells/ml.
During the month of October he was hospitalized in Catanzaro Hospital for diagnostic controls.

Viral load was of 18,000 copies/ml
CD4 cell count was 701 cells/ml.

The epidemiological investigation did not identify any known risk factors for HIV infection (i.e., drug use, tattoos, homosexual or heterosexual contact, biological fluid exposure). **No rape**

Thus, we tried to reconstruct the epidemiological network using the pol viral sequences (1271 nucleotides) detected by a sample collected in November 2009, before starting antiretroviral therapy.

Phylogenetic analysis classified our pol gene sequence as a ‘‘nonpure’’ B subtype
Using a second data set containing more than 200 B subtypes sequences spreade in europe and in the middle east asia showed that the patient’s isolate was closely related to B subtype sequences from Spain, forming a significant monophyletic cluster (bootstrap value >70%)
SimPlot and split-decomposition analyses showed the sequence as a possible BFB recombinant form; however, we classified it as ‘‘BUB’’ (U for unclassified) since the F trait and the break-point of recombination were under the threshold value (70%) useful for statistical significance (the tree was simplified cutting sequences not closely related)
The young man was enrolled in the peace military mission of the ISAF (International Security Assistance Force). In this context, soldiers from different nations (Italy, the United States, and Spain) were living inside the same logistic base for periods ranging from 2 to 9 months. After this period soldiers affected by depression usually spend 12–15 days with their family or 5 days in Abu-Dabhi where they usually meet people from different Countries (sex workers).
when the soldier returned in hospital on February 11, 2010 we tried to obtain more detailed information about possible sexual contacts in the different Settings in a

**friendly discussion**

At this point, the Italian soldier remembered an occasional sexual contact he had with a young Spanish woman in May 2009 during a vacation period spent in Italy.
He also reported having had an episode of fever that lasted 4 days, with fatigue and a sore throat, in June 2009, suggesting primary HIV infection. Unfortunately, we could not trace the woman involved to obtain clinical samples to compare the sequences.

However, it is still remarkable how in this case phylogenetic driven epidemiological evidence allowed us to determine the most probable source of HIV infection.
HIV-1 subtype C transmission network: The phylogenetic reconstruction strongly supports the epidemiological data

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2. Case description

Briefly, a nurse (S1) was infected by a needle stick injury while sampling blood from an infant (S2) born from a HIV-1 seropositive mother (S3) unaware of her seropositivity, and her husband, originating from Zimbabwe, later certified as HIV-infected (S4). All the specimens from the patients enrolled in our study were collected between February and July 2007.
To support the epidemiological investigation, we reconstructed the epidemiological network within a calendar timescale, using the Pol viral sequences (918 nucleotides) isolated from all the subjects (S1–S4) enrolled, (father, mother, kind and nurse) before initiating any antiretroviral therapy.
The index case (S4) was at the root of the probable transmission cluster, followed by patients S3, S2 and S1. The tMRCA of the network was estimated to be at a mean 4.5 years (95% HPD: 1.5–7.7 years) before July 2007, corresponding to late 2002 and early 2003.

Our analysis not only confirmed the probability of an epidemiological network but allowed us to date the transmission events with good approximation.

In addition to the existence of an epidemiological relationship among the four patients studied, the dated tree supported the time point and direction of each transmission, estimated on the basis of the phylogeny, and agreed with the presumptive time of infection on the basis of clinical history-taking.

In particular, our analysis confirm that S4 was the index case and probably acquired the infection about 4.5 years before sampling (2002/03 year), even if he verbally reported a negative HIV-1 test in 2004.
The mother’s (S3) node tMRCA dates back to the mid 2006. Detailed interview showed that S4 and S3 met in May 2006 and pregnancy was diagnosed one month later when the woman suffered an influenza-like syndrome, suggesting that the estimated tMRCA might correspond to the period in which the woman acquired the infection.

Lastly, the delivery of the baby (February 2007) might correspond to the origin of the node connecting the baby’s isolate with that obtained by the nurse exposed to the baby’s blood two weeks later.

The sequence and times of the transmission events, estimated on the phylodynamic reconstruction of the transmission network within a calendar timescale, broadly in agreement with the epidemiological data, significantly improved the investigation.

Lastly, we strengthen the concept that phylogeny can be an important way of tracing epidemiological relationships in cases with unknown links.
Fig. 1. Bayesian phylogenetic tree of 49 HIV-1 subtype C Pol sequences implementing relaxed molecular clock. S1–S4 patients' transmission cluster is evidenced in bold. * represent the posterior probability >80%.
Serum samples were collected from 25 patients with chronic HCV, attending the University Hospital of Catanzaro during the period 2003–2009. All patients were Italians and came from different areas of Calabria.
METODI

A first dataset was used for genotyping the HCV Italian isolates and consisted of 68 HCV NS5B specific reference sequences plus 25 HCV NS5B sequences of Italian isolates (from Calabria region).

A second dataset was built to prove that Italian clusters were not split by foreign sequences and consisted of 183 HCV 4d NS5B reference sequences, plus 19 HCV 4d NS5B sequences of Italian isolates (from Calabria region).

A third dataset was used to estimate the evolutionary rate and included all available dated 4d HCV NS5B sequences collected between 1990 and 2006 plus 19 HCV NS5B sequences of genotype 4d from Italian patients collected between 2003 and 2009.

fourth dataset, was used for the reconstruction of HCV 4d time-scaled phylogeny and for population dynamic analysis with fixed evolutionary rate computed in the third data set. It included only the 19 HCV NS5B genotype 4d Italian isolates (from Calabria region) collected between 2003 and 2009.
19 (76%) were classified as genotype 4d, 2 (8%) as genotype 4a, 1 (4%) as genotype 1b and 3 (12%) as genotype 2a.
<table>
<thead>
<tr>
<th>Patient code</th>
<th>Provinces</th>
<th>Risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>c11</td>
<td>Crotone</td>
<td>N.A.</td>
</tr>
<tr>
<td>c13</td>
<td>NA</td>
<td>Intravenous drug user</td>
</tr>
<tr>
<td>c15</td>
<td>Crotone</td>
<td>Surgical intervention</td>
</tr>
<tr>
<td>c16</td>
<td>Crotone</td>
<td>NA</td>
</tr>
<tr>
<td>c17</td>
<td>Catanzaro</td>
<td>Tattoo</td>
</tr>
<tr>
<td>c45</td>
<td>Catanzaro</td>
<td>Intravenous drug user</td>
</tr>
<tr>
<td>c47</td>
<td>Reggio Calabria</td>
<td>Dental therapy</td>
</tr>
<tr>
<td>c48</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>c49</td>
<td>Catanzaro</td>
<td>Intravenous drug user</td>
</tr>
<tr>
<td>c51</td>
<td>Catanzaro</td>
<td>Intravenous drug user</td>
</tr>
<tr>
<td>c52</td>
<td>Catanzaro</td>
<td>Dental therapy</td>
</tr>
<tr>
<td>c53</td>
<td>Catanzaro</td>
<td>Blood transfusion</td>
</tr>
<tr>
<td>c57</td>
<td>Cosenza</td>
<td>NA</td>
</tr>
<tr>
<td>c58</td>
<td>Cosenza</td>
<td>HCV infected partner</td>
</tr>
<tr>
<td>c62</td>
<td>Vibo Valentia</td>
<td>Blood transfusion</td>
</tr>
<tr>
<td>c63</td>
<td>Catanzaro</td>
<td>Intravenous drug user</td>
</tr>
<tr>
<td>c65</td>
<td>Crotone</td>
<td>Blood transfusion</td>
</tr>
<tr>
<td>c66</td>
<td>Crotone</td>
<td>Dental therapy</td>
</tr>
<tr>
<td>c67</td>
<td>Vibo Valentia</td>
<td>Blood transfusion</td>
</tr>
</tbody>
</table>

NA: not available.
Supplementary Fig. 1. Maximum Likelihood tree of HCV NS5B sequences. The dataset included 183 HCV 4d subtype reference sequences downloaded from Los Alamos database (http://www.hcv.lanl.gov/) and the 19 Italian 4d isolates (named with a number and letter c). Branch lengths were drawn to scale with the bar at the bottom indicating 0.06 nucleotide substitutions per site. One * along the branch represents statistical support (Bootstrap values >70%) for the clade subtending that branch.

254x190mm (96 x 96 DPI)
the sub-clade dated to 1972 contained sequences from patients not reporting intravenous drug user as risk factor all Italian patients reporting intravenous drug users as primary risk factor grouped in the sub-clade dated to 1974.

During the 1930s, emigration to the colonies was encouraged as a solution to the problems of South Italy's population growth.

In 1938-1939, Italians were 89,098 in Lybia, 75,000 in Eritrea and nearly 20,000 in Somalia. From 1936 to 1941 Italians in Ethiopia were roughly 300,000, one-third of these were military personnel.

They returned to their villages of origin at the end of the 2nd World War, likely introducing genotype 4 in local communities
The growth of the genotype 4d Italian epidemic was not gradual. It was maintained in a steady non-expanding phase until late 1970s, likely by sporadically acquired infections. After that, it grew exponentially between 1975 and 1990. The exponential growth of started at least 15 years after the Italian HCV incidence peak and after the broad introduction of disposable syringes in 1975. Exponential growth was probably sustained by the vast increase in blood transfusions and the spread of illicit intravenous drug user which peaked in Europe in late 1960s. The decline of the epidemic growth around 1990s coincides with the availability of safer blood products and blood transfusions since 1994.
A Case of Italian HIV Type 2 Infection: A Genetic Analysis

Massimo Ciccozzi,1 Muhammed Babakir-Mina,2 Eleonora Cella,1 Ada Bertoli,3 Alessandra Lo Presti,1 Janak K. Maniar,4 Carlo Federico Perno,2,3 and Marco Ciotti2

Human immunodeficiency virus type 2 (HIV-2), originally restricted to Western Africa, is now spreading to Western European countries because of migration from endemic areas. Therefore, it is mandatory to enforce the surveillance and improve the diagnostics of this neglected infection. In this report, we describe a case of HIV-2 infection affecting an Italian citizen along with three cases from India. Phylogenetic analysis showed that the viral strain identified in the Italian patient clustered with a strain isolated from an immigrant living in France. Of the three Indian strains, two clustered together and were statistically supported, whereas one clustered with a strain from Guinea Bissau. The description of the first case of HIV-2 infection in an Italian citizen indicates that the virus is spreading from endemic areas to countries involved in migration. A strict monitoring and improvement of the diagnostic molecular tools are necessary to avoid misdiagnosis with relevant clinical consequences.
Real-time PCR and sequencing analysis

HIV-1 was searched by real-time PCR using the Abbott RealTime HIV-1 assay according to the manufacturer's instructions (Abbott molecular, Des Plaines, IL). Plasma RNAs were run on the m2000 system, a platform capable of automated RNA extraction and PCR set-up, followed by amplification/detection.

HIV-2 was instead detected by an HIV-2 Real Time RT-PCR kit (Shanghai ZJ Bio-tech Co., Ltd., Shanghai, China). Plasma samples were extracted by a QIAamp Viral RNA Mini kit (Qiagen, Milan, Italy) and 5 l were added to the PCR mix according to the indications of the manufacturer. Positive samples were further characterized by amplification and sequencing of a 546-bp fragment of the HIV-2 V3 region.

Phylogenetic analysis

The HIV-2 env sequence (V3 region) of four patients (IT1, IN 1, 2, and 3) were aligned and compared using two different data sets: (1) reference sequences downloaded from the Los Alamos database (http://www.hiv.lanl.gov/content/index) and (2) sequences downloaded from the NCBI database with similarity > 90% (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences in both data sets were aligned using CLUSTAL X; then the sequences were manually edited with the Bioedit program and gaps were removed from the final alignment. The accession numbers of the sequences used are listed in the phylogenetic tree of Fig. 1.

The phylogenetic tree was constructed using the PAUP package. We employed the general reversible model (HKY) of nucleotide substitution, incorporating maximum likelihood (ML) estimates of base composition and the shape parameter ($\alpha$) of a gamma distribution ($\gamma$) model of among-site rate variation as it consistently gave much higher likelihood values using Modeltest v.3.7 implemented in PAUP. The maximum likelihood tree was estimated under this model using tree bisection-reconnection (TBR) branch swapping. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed through a bootstrap analysis using 1000 replicates for the neighbor-joining (NJ) tree and through the zero branch length test for the ML tree.

The tree was rooted with a midpoint rooting.

Nucleotide sequences accession number

The nucleotide sequences obtained with this study have been deposited in GenBank under the following accession numbers: JF717827–JF717830.

**FIG. 1.** Phylogenetic relationships of the Italian and Indian HIV-2 isolates with the subtype-specific reference sequences downloaded from the Los Alamos sequence database (www.hiv.lanl.gov/content/index). The reference sequences used in the analysis are shown in the tree with their original accession numbers. The asterisks (*) along a branch represent significant statistical support for the clade subtending that branch ($p=0.001$ in the zero-branch-length test; bootstrap support 75%). The scale bar indicates 0.09 nucleotide sequence divergence.
The circulation of HIV-2 is mainly restricted to countries of West Africa. However, immigration from this African region has led to the spread of the virus to other continents with most of the cases diagnosed in France and Portugal.

In Italy, the attention in regard to this neglected infection recently increased, especially among immigrants.

we describe the first Italian case of HIV-2 infection along with three cases from India. A phylogenetic analysis was carried out to determine the genetic features of these four HIV-2 strains. Of the three Indian strains, two were highly related, suggesting that they shared the same source of infection.

The third sequence clustered with a strain from Guinea Bissau. However, the similarity between this Indian strain and the Guinea Bissau strain is not suggestive of a recent common ancestor. About the Italian strain, it clustered with a sequence isolated in France. It seems that the common ancestor of these two viruses was in Africa with two importations into Europe, indicating that this clade of viruses has been evolving in France and Italy for decades. Likewise, the phylogeny cannot indicate the direction of travel, and it is as likely that the French virus came through Italy as it is that the Italian virus came through France.
An epidemiological investigation in reconstructing a probable transmission network: a case report

Eugenio Nelson Cavallari¹, Eleonora Cella², Claudia Montagna³, SaraSerafino¹, Pietro Vittozzi¹, Alessandra Lo Presti², Laura Mazzuti³, Ombretta Turriziani³, Giancarlo Ceccarelli¹, Gabriella d’Ettorre¹, Vincenzo Vullo¹, Massimo Ciccozzi

To reconstruct the transmission network between two HIV-1 infected subjects, a phylodinamic analysis was conducted. The dated tree allowed to date the transmission event, the time point and direction of the transmission estimated on the basis of the phylogeny, and agreed with the presumptive time of infection on the basis of clinical history-taking.

We recently described a case of an HIV subtype C transmission by a needle stick injury in a nurse from an infant, and a case of HIV-1 infection with a BFB putative recombinant form in a 24-year-old Italian soldier in service in Kabul, Afghanistan. In both these cases was underlined the importance of the phylogenetic and phylodinamic analysis to support the epidemiological investigation in reconstructing the transmission network.

In the present case report, a case of "parental" transmission between a man and a women living together was described to mark once more the importance of the “tightened collaboration” between phylogeny and epidemiology.
EPIDEMIOLOGICAL INVESTIGATION

At the time of their presentation to our clinical center the male was 30 years old and the female was 21 years old.

He became addicted to injective drugs in 1996 by the age of 14 years old and reached the rehabilitation community after 14 years of drug abuse, during which he was used to needle exchange practice.

She was addicted to injective drugs from the age of 12 years old until the age of 19 years old, she also practiced needle exchanges;

they knew each other when she reached his same rehabilitation community.

At the beginning of April 2013 he came to our attention because of a suspected infection with Hepatitis C Virus (HCV).

The patient reported a virulent and recent episode of shingles of the right hemi-thorax. At the physical examination we noticed the presence of several genital lesions suggestive of condylomas.
On the basis of the epidemiological history and these clinical findings, we proposed the patient to be tested for HIV infection. He was found to be HCV IgG positive and HCV RNA negative.

He resulted HIV-Ag/Ab positive with an HIV RNA of 102,900 copies/ml and a CD4+ T cells count of 20 cells/µL.

Due to this novel findings, the girlfriend of the patient was proposed to test for HIV and HCV infections. She resulted HIV-Ag/Ab negative, HCV IgG positive and HCV RNA negative.

After 2 weeks the woman presented to the emergency Department of an urban hospital for elevated fever and skin rash on every part of the body. The patient was discharged with diagnosis of “Viral Infection” and with the indication to present in Infectious diseases ambulatory.

The day after the disclosure the patient arrived to our centre and she reported a delay of two days in the period. She was tested for β-HCG, that resulted positive. The research for HIV-Ag/Ab was repeated and resulted negative again. As stated by the DHHS guidelines, in consideration of an HIV RNA <10,000 copies/ml with a negative HIV-Ag/Ab test, we repeated the HIV RNA test on a different specimen from the same patient and found an HIV RNA of 1,302,000 copies/ml.
At the beginning of May she experienced a spontaneous abortion due to acute retroviral syndrome. To support the epidemiological investigation, we reconstructed the transmission network within a calendar timescale on the basis of a recently described phylogenetic-statistical framework using the env viral sequences from the two subjects.

Our patient’s isolates formed a significant monophyletic cluster (posterior probability = 1) showing a strong relationship and affirming to have infected by the same virus.

In the male dating the male sequence was in relationship with a sequence from USA and the tMRCA (Time to Most Recent Common Ancestor) was estimated to be of 1998 (95% HPD: 1991-2004).
Co-circulation of Dengue and Chikungunya Viruses, Al Hudaydah, Yemen, 2012

Giovanni Rezza, Gamal El-Sawaf, Giovanni Faggioni, Fenicia Vescio, Ranya Al Ameri, Riccardo De Santis, Ghada Helaly, Alice Pomponi, Dalia Metwally, Massimo Fantini, Hussein Qadi, Massimo Ciccozzi, and Florigio Lista

Vectorborne infections are not uncommon in the Middle East (1). In particular, recurrent outbreaks of dengue fever have been reported on the Arabian Peninsula since 1990 (2). In Yemen, dengue virus (DENV) infections have reemerged with higher frequency during the last decade (3); in 2010, during a dengue outbreak that occurred in the southern governorate of Hadramout (4), cases of dengue hemorrhagic fever were identified (5). In 2010–2011, another mosquitoborne virus, the chikungunya virus (CHIKV), was detected in febrile patients in Al Hudaydah, Yemen (6). To evaluate to what extent these arboviruses are involved in dengue-like illness outbreaks in Yemen, we conducted a study in Al Hudaydah.
Figure 1. Location of Al Hudaydah, Yemen, where the co-circulation of dengue virus, chikungunya virus, and other dengue-like viruses was studied in 2012. Other important towns, Sanaa, Aden, and Al Mukalla (the capital of Hadramout governorate), are also shown.
### Table 1. Dengue virus–positive study participants by diagnostic category, Al Hudaydah, Yemen, 2012*

<table>
<thead>
<tr>
<th>Study participants</th>
<th>IgM+/PCR−, recent infection</th>
<th>PCR+/IgM−, acute infection</th>
<th>IgM+/PCR+, acute infection</th>
<th>IgM+ and/or PCR+, acute or recent infection</th>
<th>IgM−/PCR−, no acute or recent infection</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total†</td>
<td>61 (15.2)</td>
<td>44 (11.0)</td>
<td>11 (2.7)</td>
<td>116 (29.0)</td>
<td>284 (71.0)</td>
<td>400 (100.0)</td>
</tr>
<tr>
<td>Positive for dengue virus IgG‡</td>
<td>48 (78.7)</td>
<td>22 (50.0)</td>
<td>6 (54.5)</td>
<td>76 (65.5)</td>
<td>214 (75.3)</td>
<td>290 (72.5)</td>
</tr>
</tbody>
</table>

*+, positive; −, negative.
†The denominator is represented by the total study population.
‡The denominator is represented by the number of individuals in each category.

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**Figure 2. Trends for cases of dengue virus, chikungunya virus, and other dengue-like viruses, Al Hudaydah, Yemen, 2012.**

A) Number of cases by month. B) Monthly percentages of cases by virus type.
Phylogeny of Dengue and Chikungunya viruses in Al Hudayda governorate, Yemen

Massimo Ciccozzi a,b,c, Alessandra Lo Presti a, Eleonora Cella d, Marta Giovanetti a, Alessia Lai c, Gamal El-Sawaf d, Giovanni Faggioni e, Fenicia Vescio a, Ranya Al Ameri f, Riccardo De Santis e, Ghada Helaly d, Alice Pomponi e, Dalia Metwally d, Massimo Fantini g, Hussein Qadi f, Gianguglielmo Zehender c, Florigio Lista e, Giovanni Rezza a

Yemen, which is located in the southwestern end of the Arabian Peninsula, is one of countries most affected by recurrent epidemics caused by emerging vector-borne viruses. Dengue virus (DENV) outbreaks have been reported with increasing frequency in several governorates since the year 2000, and the Chikungunya virus (CHIKV) has been also responsible of large outbreaks and it is now a major public health problem in Yemen. We report the results of the phylogenetic analysis of DENV-2 and CHIKV isolates (NS1 and E1 genes, respectively) detected in an outbreak occurred in Al-Hudayda in 2012. Estimates of the introduction date of CHIKV and DENV-2, and the phylogeographic analysis of DENV-2 are also presented.
Phylogenetic analysis showed that the Yemen isolates of DENV belonged to the lineage 2 Cosmopolitan subtype,
CHIKV isolates from Yemen belonged to the ECSA genotype.
CHIKV isolates from Yemen were statistically supported and dated back to the year 2010 (95% HPD: 2009–2011); these sequences showed an alanine in the aminoacid position 226 of the E1 protein.
Phylogeographic analysis of DENV-2 virus showed that cluster 1, which included Yemen isolates, dated back to 2003 Burkina Faso strains (95% HPD 1999–2007). The Yemen, cluster dated back to 2011 (95% HPD 2009–2012).
HIV forensics: pitfalls and acceptable standards in the use of phylogenetic analysis as evidence in criminal investigations of HIV transmission

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1NAM, London, UK, 2National AIDS Trust, London, UK, 3Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium, 4Research Institute for Law, Politics and Justice, Keele University, Staffordshire, UK, and 5Department of Virology, Royal Free Hospital and Royal Free & University College Medical School, London, UK
Pitfalls

Phylogenetic analysis of HIV gene sequences is complex and its findings do not achieve the levels of certainty obtained with the forensic analysis of human DNA.

Although two individuals may carry HIV strains that are closely related, these will not necessarily be unique to the two parties and could extend to other persons within the same transmission network.

Two facts need to be proved:

(1) that the defendant infected the complainant, and
(2) that the defendant was ‘reckless’ (i.e. that at the relevant time he or she was aware of the risk of infecting the complainant).
Thus, the appropriate interpretation would include the following questions.

Have the appropriate controls been included?

Are the two viruses more closely related to each other than to the controls?

Is there anybody else infected with the virus that is, or could be, also related?

Is there any other epidemiological evidence of linkage between individuals?
The viral sequences from the two subjects display a high level of similarity and are more closely related to each other than to other strains circulating in a population with the same epidemiological profile.

The possibility that an unknown third person might be involved, lead in the direction of transmission that cannot be proven.
The story

In May 1998, the Al-Fateh Children’s Hospital (AFH) in Benghazi, Libya noted their first case of HIV-1 infection.

In September 1998, another 111 children who had been admitted to the hospital were found to be HIV-1 positive.

The outbreak was reported by local hospital authorities and representatives from the World Health Organization (WHO) were sent to AFH in December 1998 to examine the cause of the infections.

The resultant WHO report suggests that there were multiple nosocomial HIV-1 infections at AFH. The report also notes the lack of required medical equipment in the hospital.
In total 418 children were infected with HIV-1 in the AFH outbreak.

248 (59.3%) of these children were sent to hospitals in Geneva, Rome and Milan for care, treatment and virological assessment.

Epidemiological data, available for 37 of the children, indicated that all of these children had undergone invasive procedures while in the hospital, or as outpatients.

At the time of first observation, 216 (87.1%) of the children were asymptomatic, or mildly symptomatic; 29 (11.7%) had moderately severe symptoms, and 3 (1.2%) had severe symptoms according to the CDC classification system.

Serological testing of a subset of plasma specimens indicated that 75 (43.1%) of 174 children were co-infected with HCV.
In March 1998 six foreign doctors (five Bulgarian nurses and a medical doctor from Palestine) joined the medical staff at AFH.

One year later, these individuals were accused of purposefully infecting more than 400 children with HIV-1. They have been detained in prison (tortured) ever since.

Epidemiological investigation
there is a single introduction???????

In April 2003, at the court’s request, two international HIV/AIDS scientists, Luc Montagnier and Vittorio Colizzi conducted a scientific inquiry into the Benghazi outbreak. In their report, they conclude that given the high rate of Hepatitis B and C infection amongst the children, the contamination was more likely to be caused by pre-existing poor hygiene practices rather than a single introduction.

Stiring up the hell
In May 2004, the foreign medical staff were imprisoned, tortured, accused of terrorist act, and then condemned to death.

However, in response to international appeal, the Libyan Supreme Court ordered a retrial on the 25th of December 2005.

The new trial began in Tripoli on the 11th of May 2006, and on the 29th of August, the prosecution again called for the medics to be sentenced to death.

The last session of the trial began on the 4th of November 2006 and the final verdict was for the 19th of December.

Attorneys from *Lawyers without Borders*, who represented the defendants, have appealed to international AIDS experts to conduct an independent scientific inquiry into the history of the Benghazi HIV-1 outbreak.

The paper written was a response to their appeal.
Study Populations:
The demographic and clinical data for the subset of 44 children available for HIV-1 sequence analysis was comparable to that of the entire European cohort.

Thirtythree (75.0%) of these children were asymptomatic, or mildly asymptomatic (N/A), 11 (25%) had moderately severe symptoms, and 1 (2.3%) had severe symptoms.

Twenty one (47.7%) had serological evidence of HCV co-infection.

Plasma specimens for HIV-1 gag gene analyses were collected from 44 children visiting the Bambino Gesù Children Hospital, Rome, Italy between 2000 and 2003.

61 sera sample were collected for HCV E1/E2 gene analysis from children visiting the University Hospital in Geneva between 1998-2001.
Information recorded for each patient: Demographic data Clinical history Immunologic parameters (CD4 and CD8) Virologic parameters (Viral load) Therapeutic history Genotypic mutations (protease, reverse transcriptase, integrase, gp120, gp41,)
The HIV-1 sequences from the hospital form a well supported monophyletic cluster within the CRF02_AG clade, indicating that the outbreak arose from one CRF02_AG lineage.

The cluster is closest to three west African reference sequences  the basal location of which suggests that the Al-Fateh Hospital lineage arrived in Libya from there.

The HCV sequences from the hospital formed three monophyletic clusters containing
- 11 subtype-4a sequences, phylogenetically placed among Egyptian subtype 4a lineages;
- 22 sequences most closely related to a Cameroonian genotype-4 strain;
- 24 sequences belonging to the worldwide and prevalent subtype 1a;
- 4 remaining sequences belong to genotype 4
three west African countries

Figure 1 | HIV-1 and HCV sequences from 1998 Al-Fateh Hospital (AFH) outbreak. a–c, Estimated maximum-likelihood phylogenies for HIV-1 CRF02_AG (a), HCV genotype 4 (b) and HCV genotype 1 (c). Source of sequences used for analysis: AFH, red; Egypt, green; Cameroon, blue. Black circles mark the common ancestor of HCV subtype 4a and 1a; numbers above AFH lineages give clade support values using bootstrap and bayesian methods, respectively. Scale bar units are nucleotide substitutions per site. For visual clarity, AFH clusters are represented by triangles and some non-informative reference strains are excluded.
Figure 2 | Estimated dates of the most recent common ancestor for each cluster. Results obtained by using different evolutionary models. Vertical lines show the 95% highest posterior density intervals. Red line shows time of arrival of the foreign staff in March 1998. For further details, see supplementary information. ‘Const’, constant size; ‘Expo’, exponential growth.
these results supported the existing nosocomial transmission scenario and suggest that Al- Fateh Hospital had a long-standing infection- control problem.

The earlier origin and greater number of HCV clusters than HIV-1 clusters reflect the higher transmissibility of HCV compared with HIV-1 by such routes.

we showed that the HIV-1 and HCV strains responsible were being spread and transmitted among individuals attending the hospital before March 1998, indicating that many of the transmissions giving rise to the infection clusters must have already occurred before the foreign medical staff arrived.
The Libya’s decision was to commute to life imprisonment the death sentences of five Bulgarian nurses and a Palestinian medic (that began Bulgarian citizen).

All six were imprisoned for eight years on false charges of deliberately infecting children with HIV in the hospital where they worked.

The subsequent decision by Bulgaria’s president to pardon and release the six immediately (when landed in Bulgarian territory) upon their extradition to Sofia.

The 17 July announcement that Libya’s Higher Judicial Council had commuted the death sentence to life imprisonment should have been accompanied by an explicit acknowledgement that the real cause of the outbreak was an accident stemming from insufficient infection controls and hospital safety precautions.
What is the real tree?

The answer is

“a real tree is like the truth, that is something that is found in a fund of a well that do not have a fund”

San Patrizio well

*Antonio da Sangallo il Giovane*
A total of 9936 confirmed, probable, and suspected cases of Ebola virus disease (EVD) have been reported in five affected countries (Guinea, Liberia, Sierra Leone, Spain, and the United States of America) and two previously affected countries (Nigeria and Senegal) up to the end of 19 October. A total of 4877 deaths have been reported.

The outbreaks of EVD in Senegal and Nigeria were declared over on 17 October and 19 October 2014, respectively.
<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>Ebolavirus species</th>
<th>Cases</th>
<th>Deaths</th>
<th>Case fatality</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Democratic Republic of Congo</td>
<td>Bundibugyo</td>
<td>57</td>
<td>29</td>
<td>51%</td>
</tr>
<tr>
<td>2012</td>
<td>Uganda</td>
<td>Sudan</td>
<td>7</td>
<td>4</td>
<td>57%</td>
</tr>
<tr>
<td>2012</td>
<td>Uganda</td>
<td>Sudan</td>
<td>24</td>
<td>17</td>
<td>71%</td>
</tr>
<tr>
<td>2011</td>
<td>Uganda</td>
<td>Sudan</td>
<td>1</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>2008</td>
<td>Democratic Republic of Congo</td>
<td>Zaire</td>
<td>32</td>
<td>14</td>
<td>44%</td>
</tr>
<tr>
<td>2007</td>
<td>Uganda</td>
<td>Bundibugyo</td>
<td>149</td>
<td>37</td>
<td>25%</td>
</tr>
<tr>
<td>2007</td>
<td>Democratic Republic of Congo</td>
<td>Zaire</td>
<td>264</td>
<td>187</td>
<td>71%</td>
</tr>
<tr>
<td>2005</td>
<td>Congo</td>
<td>Zaire</td>
<td>12</td>
<td>10</td>
<td>83%</td>
</tr>
<tr>
<td>2004</td>
<td>Sudan</td>
<td>Sudan</td>
<td>17</td>
<td>7</td>
<td>41%</td>
</tr>
<tr>
<td>2003 (Nov-Dec)</td>
<td>Congo</td>
<td>Zaire</td>
<td>35</td>
<td>29</td>
<td>83%</td>
</tr>
<tr>
<td>2003 (Jan-Apr)</td>
<td>Congo</td>
<td>Zaire</td>
<td>143</td>
<td>128</td>
<td>90%</td>
</tr>
<tr>
<td>2001-2002</td>
<td>Congo</td>
<td>Zaire</td>
<td>59</td>
<td>44</td>
<td>75%</td>
</tr>
<tr>
<td>2001-2002</td>
<td>Gabon</td>
<td>Zaire</td>
<td>65</td>
<td>53</td>
<td>82%</td>
</tr>
<tr>
<td>2000</td>
<td>Uganda</td>
<td>Sudan</td>
<td>425</td>
<td>224</td>
<td>53%</td>
</tr>
<tr>
<td>1996</td>
<td>South Africa (ex-Gabon)</td>
<td>Zaire</td>
<td>1</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>1996 (Jul-Dec)</td>
<td>Gabon</td>
<td>Zaire</td>
<td>60</td>
<td>45</td>
<td>75%</td>
</tr>
<tr>
<td>1996 (Jan-Apr)</td>
<td>Gabon</td>
<td>Zaire</td>
<td>31</td>
<td>21</td>
<td>68%</td>
</tr>
<tr>
<td>1995</td>
<td>Democratic Republic of Congo</td>
<td>Zaire</td>
<td>315</td>
<td>254</td>
<td>81%</td>
</tr>
<tr>
<td>1994</td>
<td>Cote d'Ivoire</td>
<td>Taï Forest</td>
<td>1</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>1994</td>
<td>Gabon</td>
<td>Zaire</td>
<td>52</td>
<td>31</td>
<td>60%</td>
</tr>
<tr>
<td>1979</td>
<td>Sudan</td>
<td>Sudan</td>
<td>34</td>
<td>22</td>
<td>65%</td>
</tr>
<tr>
<td>1977</td>
<td>Democratic Republic of Congo</td>
<td>Zaire</td>
<td>1</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>1976</td>
<td>Sudan</td>
<td>Sudan</td>
<td>284</td>
<td>151</td>
<td>53%</td>
</tr>
<tr>
<td>1976</td>
<td>Democratic Republic of Congo</td>
<td>Zaire</td>
<td>318</td>
<td>280</td>
<td>88%</td>
</tr>
</tbody>
</table>
COUNTRIES WITH WIDESPREAD AND INTENSE TRANSMISSION

9191 (probable, confirmed and suspected; see Annex 2) cases and 4546 deaths from EVD have been reported up to the end of 13 October 2014 by the Ministry of Health of Liberia, and 14 October by the Ministries of Health of Guinea and Sierra Leone.

A total of 9216 confirmed, probable, and suspected cases of Ebola virus disease (EVD) have been reported in seven affected countries (Guinea, Liberia, Nigeria, Senegal, Sierra Leone, Spain, and the United States of America) up to the end of 14 October. There have been 4555 deaths.

A second EVD negative sample was obtained from the single confirmed case in Senegal on 5 September (42 days ago). WHO officially declares the Ebola outbreak in Senegal over.

Following the WHO Ebola Response Roadmap structure, country reports fall into two categories: 1) those with widespread and intense transmission (Guinea, Liberia, and Sierra Leone); and 2) those with an initial case or cases, or with localized transmission (Nigeria, Senegal, Spain, and the United States of America).

An overview of the situation in the Democratic Republic of the Congo, where a separate, unrelated outbreak of EVD is occurring, is also provided (see Annex 1).

### Table 1: Ebola virus disease cases and deaths in Guinea, Liberia, and Sierra Leone

<table>
<thead>
<tr>
<th>Country</th>
<th>Case definition</th>
<th>Cases</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea</td>
<td>Confirmed</td>
<td>1217</td>
<td>671</td>
</tr>
<tr>
<td></td>
<td>Probable</td>
<td>191</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Suspected</td>
<td>111</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>1519</td>
<td>862</td>
</tr>
<tr>
<td>Liberia</td>
<td>Confirmed</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Probable</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Suspected</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>4262</td>
<td>2484</td>
</tr>
<tr>
<td>Sierra Leone</td>
<td>Confirmed</td>
<td>2977</td>
<td>932</td>
</tr>
<tr>
<td></td>
<td>Probable</td>
<td>37**</td>
<td>161**</td>
</tr>
<tr>
<td></td>
<td>Suspected</td>
<td>396</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>3410</td>
<td>1200</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>9191</td>
<td>4546</td>
</tr>
</tbody>
</table>

*Stratified data are temporarily unavailable for Liberia. **For Sierra Leone, 124 more probable deaths have been reported than have probable cases. Data are based on official information reported by Ministries of Health up to the end of 14 October 2014 for Guinea and Sierra Leone, and 13 October 2014 for Liberia. These numbers are subject to change due to on-going reclassification, retrospective investigation and availability of laboratory results.
Ebola cases are classified as suspected, probable, or confirmed depending on whether they meet certain criteria.

### Ebola case-classification criteria

<table>
<thead>
<tr>
<th>Classification</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspected</td>
<td>Any person, alive or dead, who has (or had) sudden onset of high fever and had contact with a suspected, probable or confirmed Ebola case, or a dead or sick animal OR any person with sudden onset of high fever and at least three of the following symptoms: headache, vomiting, anorexia/loss of appetite, diarrhoea, lethargy, stomach pain, aching muscles or joints, difficulty swallowing, breathing difficulties, or hiccups; or any person with unexplained bleeding OR any sudden, unexplained death.</td>
</tr>
<tr>
<td>Probable</td>
<td>Any suspected case evaluated by a clinician OR any person who died from ‘suspected’ Ebola and had an epidemiological link to a confirmed case but was not tested and did not have laboratory confirmation of the disease.</td>
</tr>
<tr>
<td>Confirmed</td>
<td>A probable or suspected case is classified as confirmed when a sample from that person tests positive for Ebola virus in the laboratory.</td>
</tr>
</tbody>
</table>
"Yambuku"
"Gueckedou"
"Makokou"
"Entsiam"n
"Kailahun"
"Etoumbi"
"BoOue"
"Mendemba"
"Etakangaye"
"Kikwit"
"Ivindo"
"Kissidougou"
"Mvoula"
"Mbandza"
"Ekata"
"Lossi"
"Ollaba"
"Luebo"
Ebola modeled glycoprotein in new epidemic. In cyan and green are shown respectively the Mucin-like and Receptor-Binding regions of GP1, in red the GP2. Van der Wall surfaces of amino acid mutated positions are shown, among them the novel mutated positions characteristics of the new epidemic are highlighted.
Impact of Ebola glycoprotein amino acid mutation in the neutralizing antibody recognition. **a)** In blue and red are shown respectively the neutralizing antibody and Ebola glycoprotein reference sequence (modified by PDB ID: 3CSY). **b)** Tridimensional alignment between Ebola glycoprotein belonging to the reference sequence (red) and with amino acid mutations characteristic of new epidemic sequences (yellow). **c)** Ebola glycoprotein and neutralizing antibody site of interaction with GP reference sequence and contribution of Ala 503. **d)** Ebola glycoprotein and neutralizing antibody site of interaction with GP sequence in the new epidemic and contribution of Val 503.
<table>
<thead>
<tr>
<th>Model</th>
<th>Free parameters</th>
<th>Log likelihood</th>
<th>Parameters estimates</th>
<th>Avg ω</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0, one ratio</td>
<td>1</td>
<td>-4083.45</td>
<td>ω= 0.399</td>
<td>0.3990</td>
</tr>
<tr>
<td>M1, neutral</td>
<td>1</td>
<td>-4069.47</td>
<td>p₀= 0.6533; p₁=0.3467</td>
<td>0.3467</td>
</tr>
<tr>
<td>M2, selection</td>
<td>3</td>
<td>-4064.66</td>
<td>p₀= 0.6587; p₁=0.3317; p₂=0.0097; ω₂= 9.5546</td>
<td>0.4309</td>
</tr>
<tr>
<td>M3, discrete</td>
<td>5</td>
<td>-4064.66</td>
<td>p₀= 0.6385; p₁=0.3513; p₂=0.0102; ω₀= 0.0000; ω₁= 0.9536; ω₂= 9.3401</td>
<td>0.4300</td>
</tr>
<tr>
<td>M7, beta</td>
<td>2</td>
<td>-4070.05</td>
<td>p= 0.00500, q= 0.00736</td>
<td>0.4000</td>
</tr>
<tr>
<td>M8, beta, and ω</td>
<td>4</td>
<td>-4064.67</td>
<td>p₀= 0.98924 ; P= 0.02093; q= 0.04029; p₁=0.01076; ω=9.04322</td>
<td>0.4284</td>
</tr>
</tbody>
</table>
Doubting of Sant Thomas

CARAVAGGIO  1571 – 1610
Oil of 107 × 146 cm realized between 1600 and 1601