6th Infectivology Today®

L'infettivologia del 3° millennio: AIDS ed altro

VI Convegno Nazionale

15- 16 -17 maggio 2014

Centro Congressi Hotel Ariston
Paestum (SA)

6th Infectivology Today®

Uso dei metodi di sequenziamento di nuova generazione in microbiologia clinica

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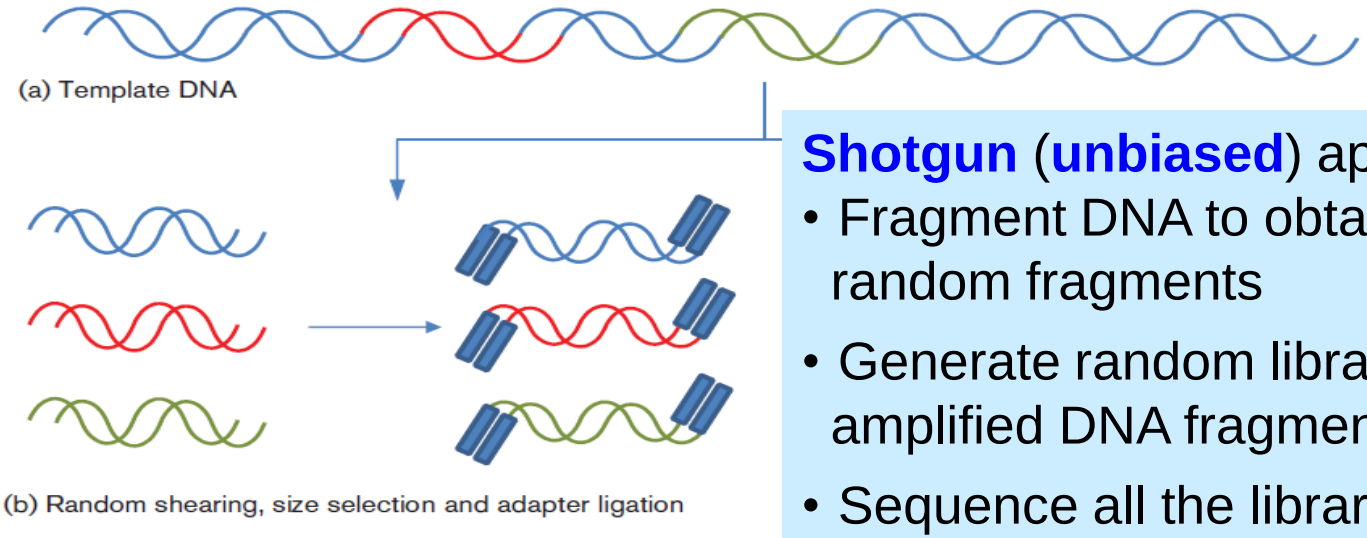
WHO Collaborating Center for clinical care, diagnosis, response and training on Highly Infectious Diseases

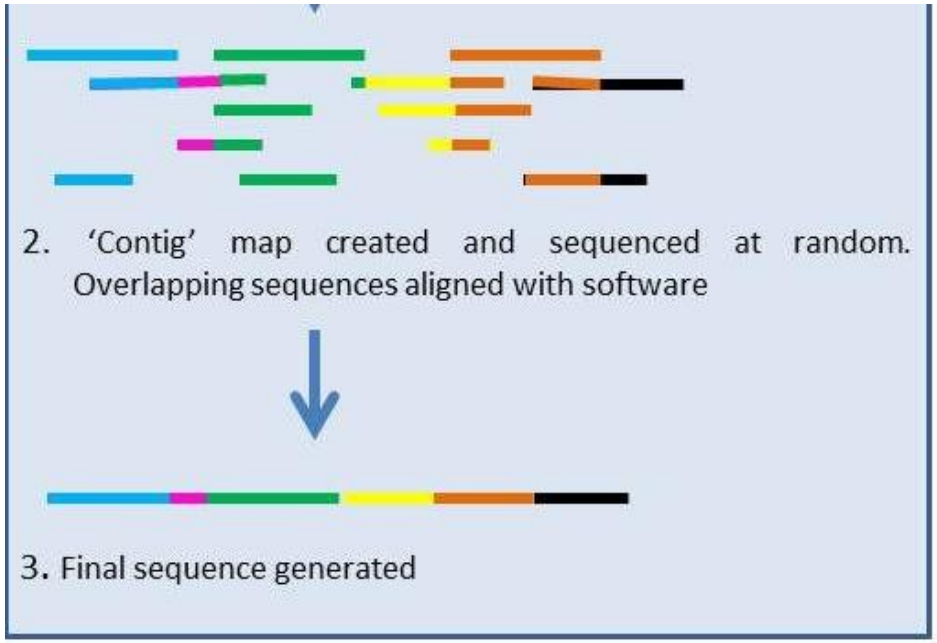
Paestum (Sa) 15- 16 -17 maggio 2014

Presentation outline

- Different NGS approaches
  - ✓Unbiased (shotgun)
  - ✓AmpliSeq
- Metagenomics
- Viral quasispecies and minority genomes
  - ✓HIV tropism
  - ✓Implications for resistance
    - HIV
    - HBV
    - HCV

Two different approaches in NGS:

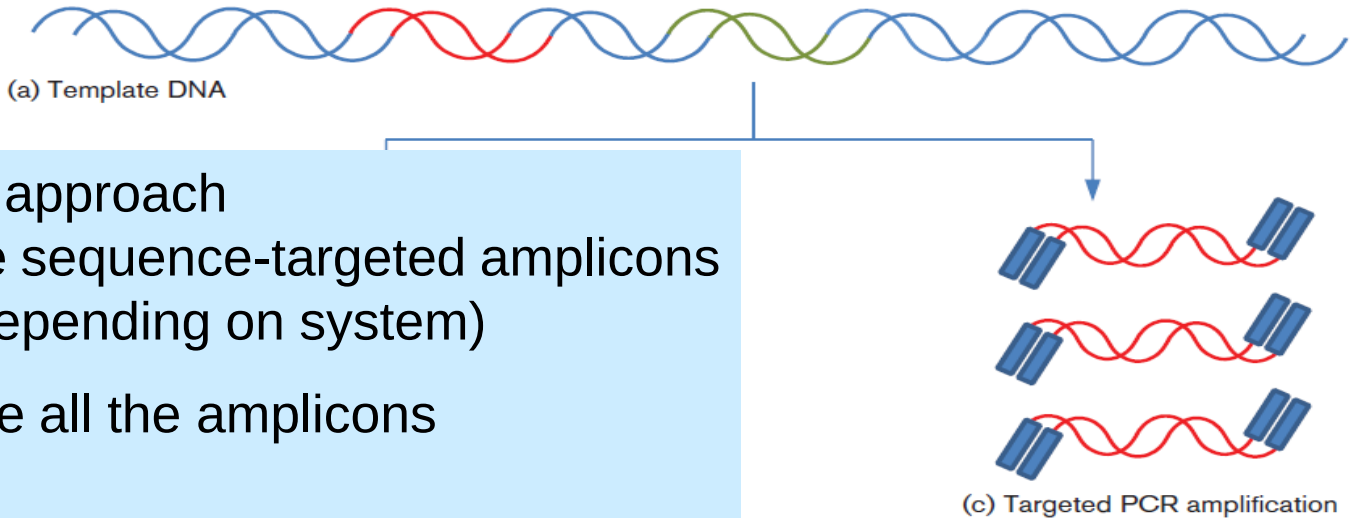




Shotgun (**unbiased**) approach

- Fragment DNA to obtain short random fragments
- Generate random library of amplified DNA fragments
- Sequence all the library fragments
- Bioinformatics elaboration:
- Alignment
- Contigs
- Blast against Genomic databases
- Classification of contigs into known or new taxonomic entities

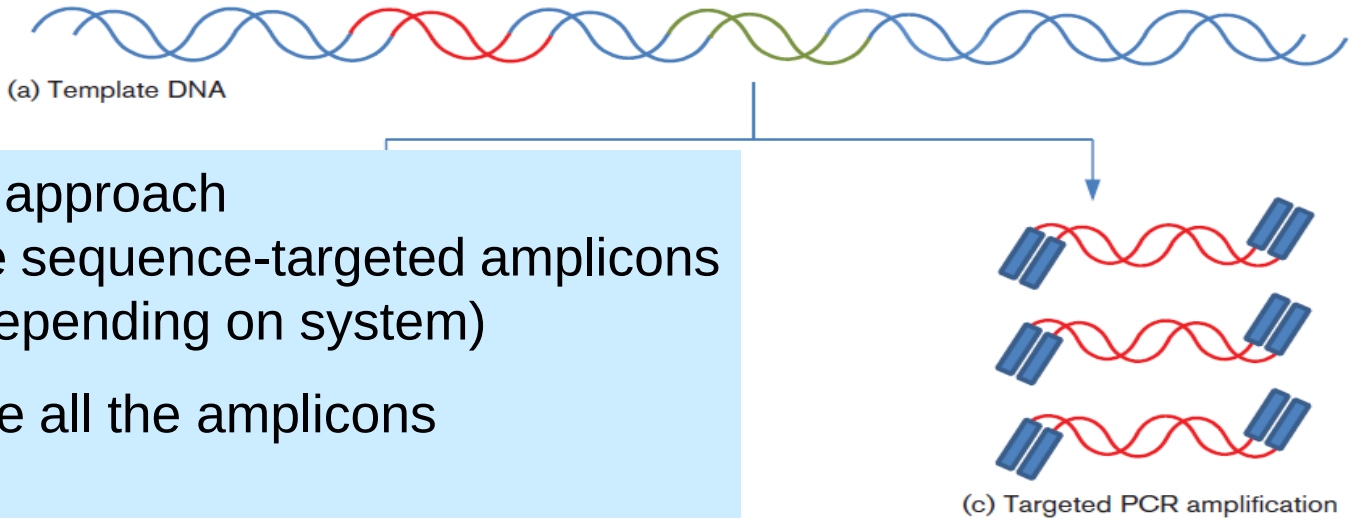
Two different approaches in NGS:



Amplicon approach

- Generate sequence-targeted amplicons (lenght depending on system)
- Sequence all the amplicons

Two different approaches in NGS:



Amplicon approach

- Generate sequence-targeted amplicons (lenght depending on system)
- Sequence all the amplicons

OPEN ACCESS Freely available online April 2010 | Volume 6 | Issue 4 | PLoS GENETICS

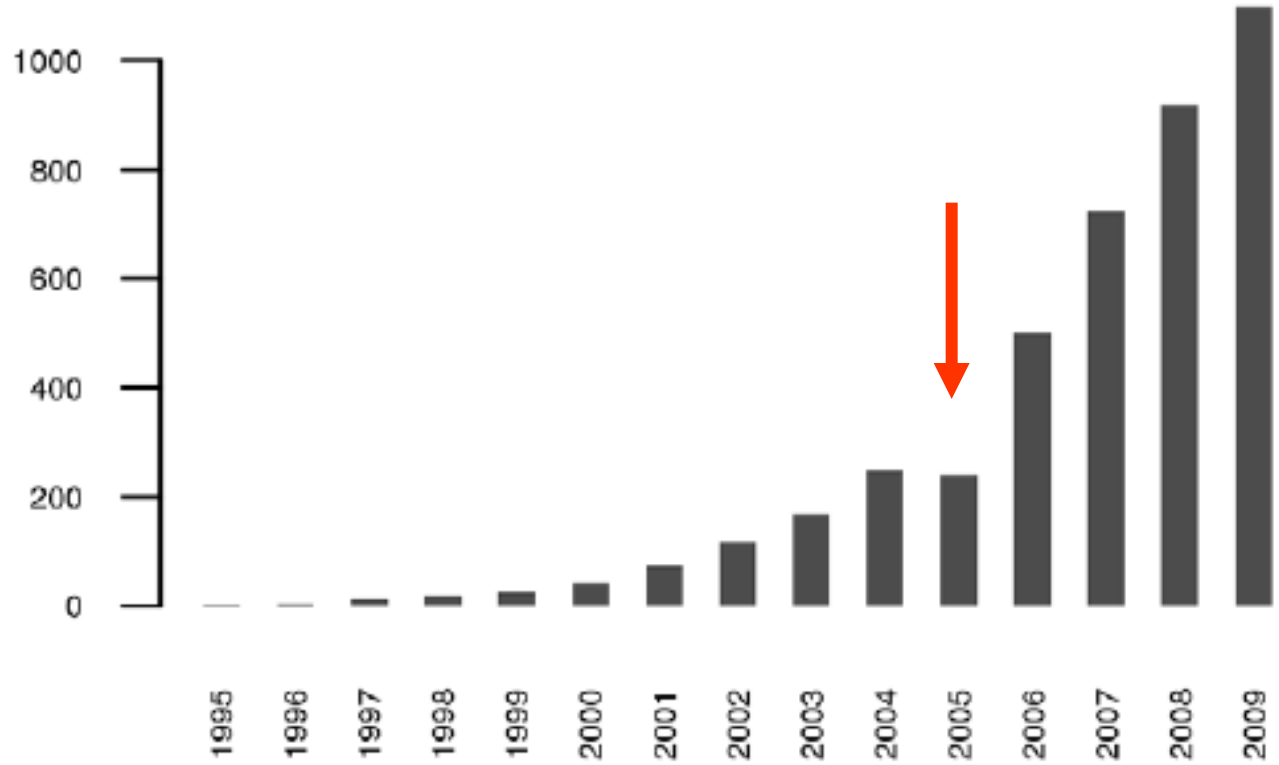
Perspective

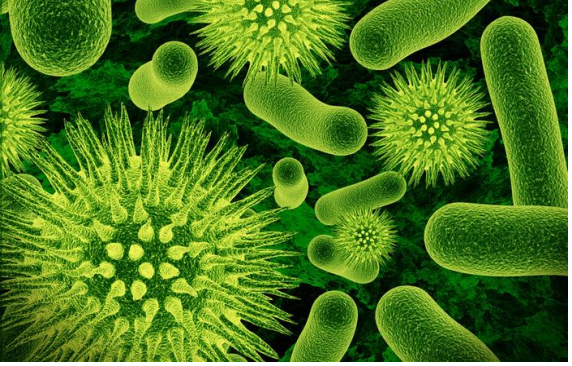
The Next Generation Becomes the Now Generation

Diego A. Martinez*, Mary Anne Nelson

Department of Biology, University of New Mexico, Albuquerque, New Mexico, United States of America

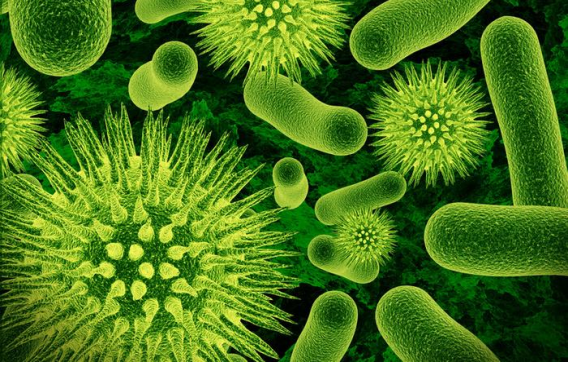
Number of genomes entered into GenBank by year as of September 2009





Application of NGS to bacterial agents

	16S rRNA (Amplicon Sequencing)
Type of information produced	The taxonomic composition and phylogenetic structure of a microbial community expressed as OTUs[8]
Application	Monitor populations
Ability to detect rare members of the community (sensitivity)	Highly sensitive. rRNA makes up 80% of total bacterial RNA
Biases	Bias produced by the probes[9] and the PCR itself[10]. The amplified region may not accurately represent the whole genome due to horizontal transfer or mutations[11].
Gene content	The gene inventory and the encoded functionality of most microbial species are largely unknown and may also vary considerably among strains.



Application of NGS to bacterial agents

	16S rRNA (Amplicon Sequencing)	Shotgun Sequencing
Type of information produced	The taxonomic composition and phylogenetic structure of a microbial community expressed as OTUs[8]	Functional and process-level characterization of microbial communities as a whole, and the reconstruction of draft genome sequences for individual community members.
Application	Monitor populations	Detect new members, new genes, and resolve complex taxonomies.
Ability to detect rare members of the community (sensitivity)	Highly sensitive. rRNA makes up 80% of total bacterial RNA	Requires much deeper sequencing to achieve the same level of sensitivity
Biases	Bias produced by the probes[9] and the PCR itself[10]. The amplified region may not accurately represent the whole genome due to horizontal transfer or mutations[11].	Sequence content bias
Gene content	The gene inventory and the encoded functionality of most microbial species are largely unknown and may also vary considerably among strains.	Generate extensive gene inventories and partial genomes. Discover new genes and biological pathways.

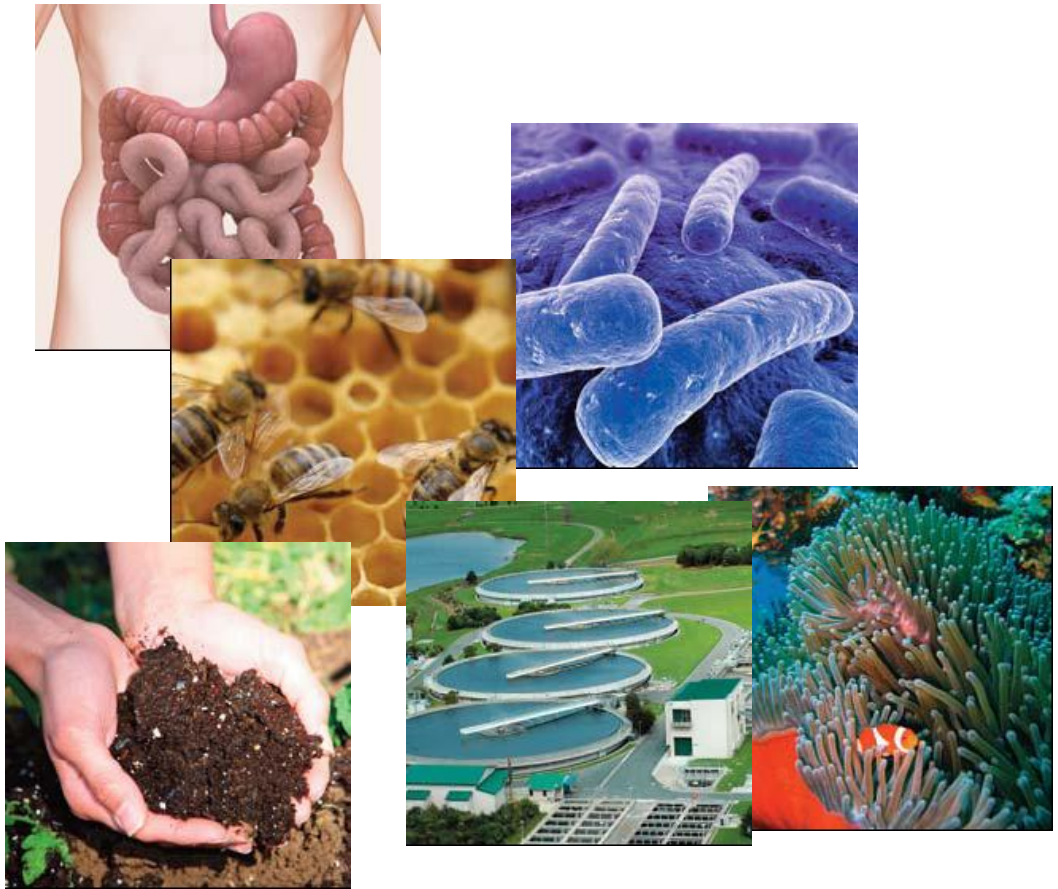
NGS applications to microbiology

- Metagenomics
  - ✓ Discovery of new pathogens
  - ✓ Molecular characterization of new/known pathogens
  - ✓ “Microbiome-Virome”
- Phylogenetic analysis
- Tracing evolutionary history
- Tracing intra-host evolution (quasispecies)
- Forensic investigation
- SNP detection
- Characterization of biological properties (e.g. HIV tropism)
- Identification of resistance markers (HIV, HCV, HBV…..)
- …….

What is Metagenomics?

Contemporary analysis of all genomes present in a given environment:

- **Serching for new species (microbial, viral…)**
- **Quantitative description of microbial communities (viroma, microbioma)**
- **Microbial variability**
- **Diagnostics…..**



- **Human body**
- **Soil samples**
- **Extreme environments**
- **Marine ecosystem**

What is Metagenomics?

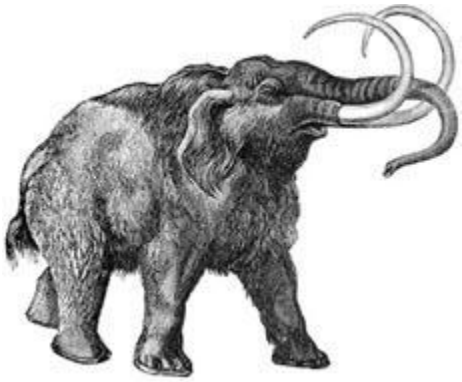
- Traditional microbial genomics
  - Sequence the genome of one organism at a time
  - Use cultures to isolate microbe of interest
- Metagenomics
  - Extract sequence data from microbial communities as they exist in nature
  - **Bypass the need for culture techniques**
    - Sequence all DNA (RNA) in sample
    - Assign sequences to Taxonomic Units (OTU)

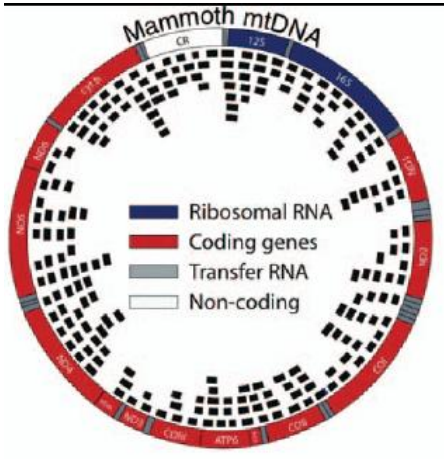
Science
AAAS

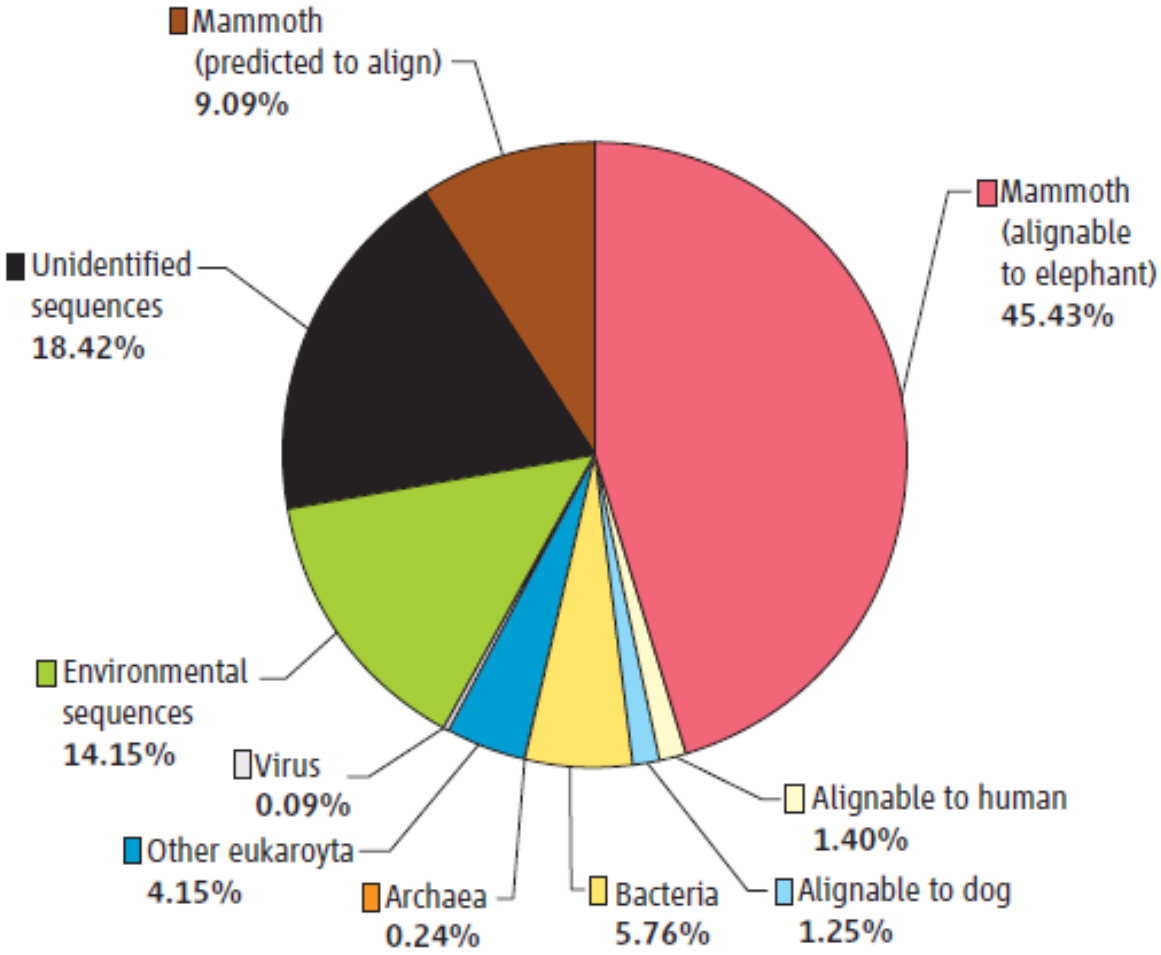
Metagenomics to Paleogenomics: Large-Scale Sequencing of Mammoth DNA

Hendrik N. Poinar, *et al.*

Science **311**, 392 (2006);
DOI: 10.1126/science.1123360







Published metagenomic studies 2002-2012

Metagenomics to Paleogenomics: Large-Scale Sequencing of Mammoth DNA
Hendrik N. Poinar, *et al.*
Science **311**, 392 (2006);
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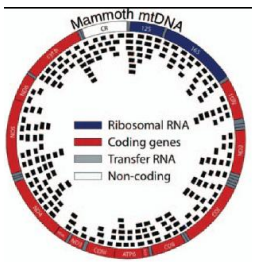
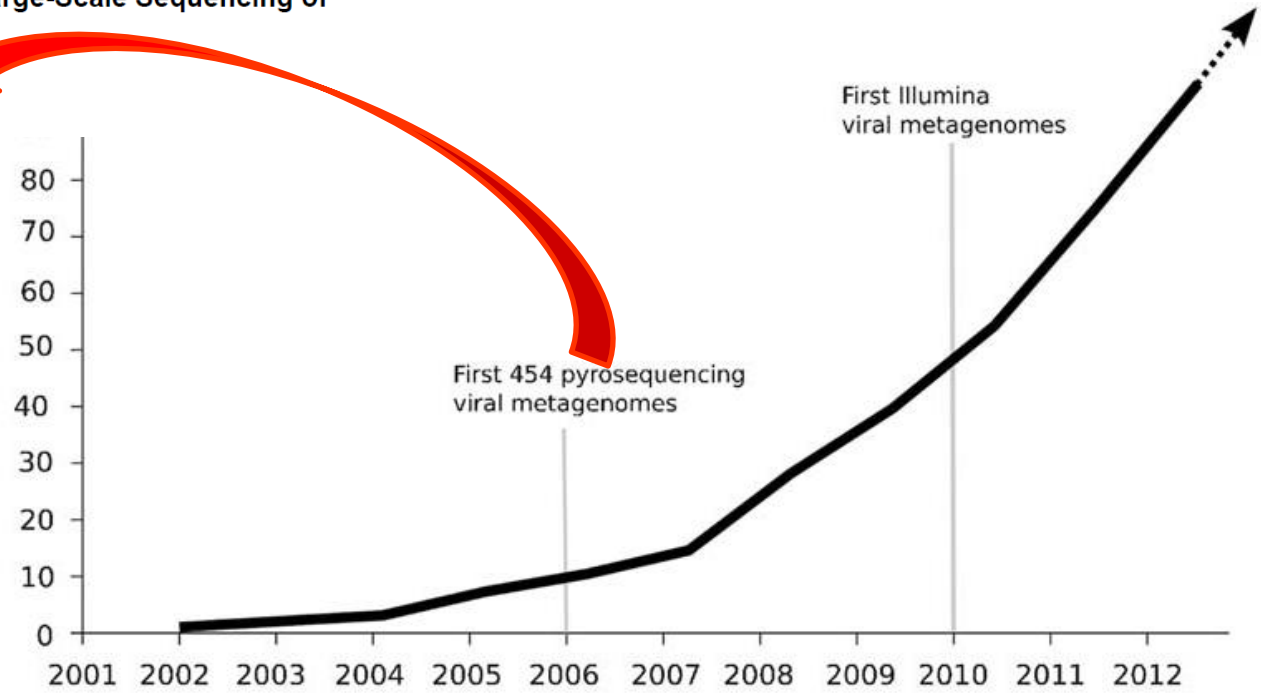
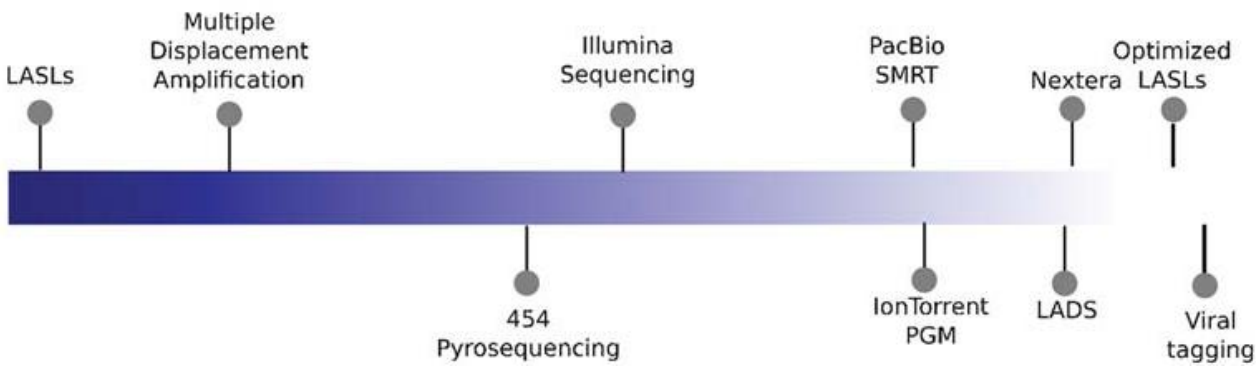






Willner D. and Hugenholtz, Bioessays 2013

Human Microbiome Project:

NIH HUMAN MICROBIOME PROJECT RESEARCHERS PUBLISH FIRST GENOMIC COLLECTION OF

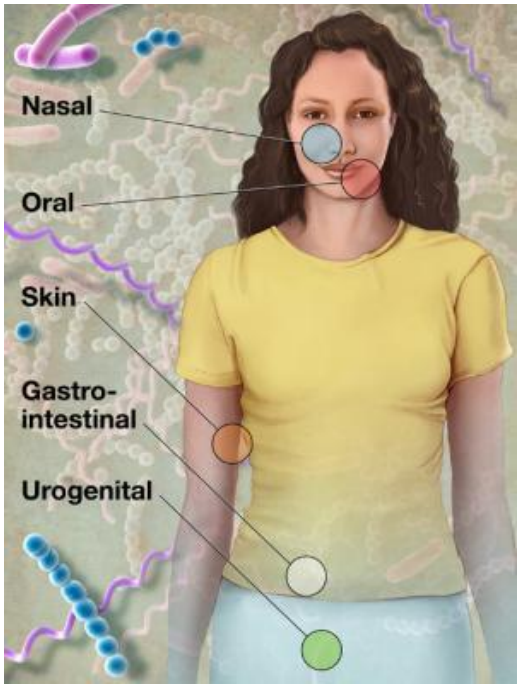
HUMAN MICROBES

Diversity of Human Microbes

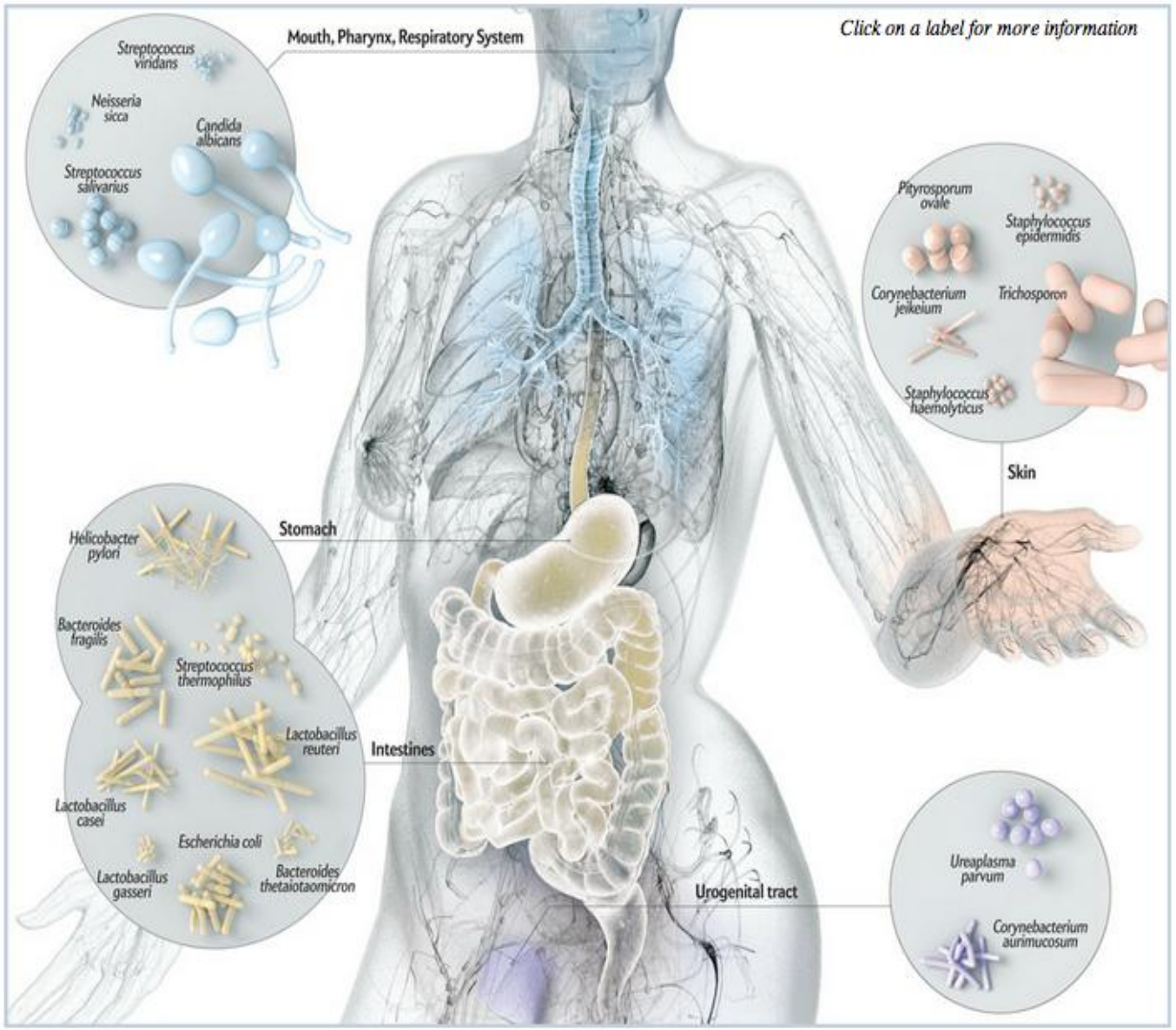
Greater Than Previously Predicted

The HMP consists of two metagenomic sequencing components:

- **generate an preliminary estimate of the complexity of the microbial community in five body sites**
- **determine whether variation in the microbiome at a site can be related to human phenotypes and/or differences between health and disease states**



the body as an ecosystem



source: Scientific American

Ecosystem

http://www.chesapeakebay.net/discover/bayecosystem

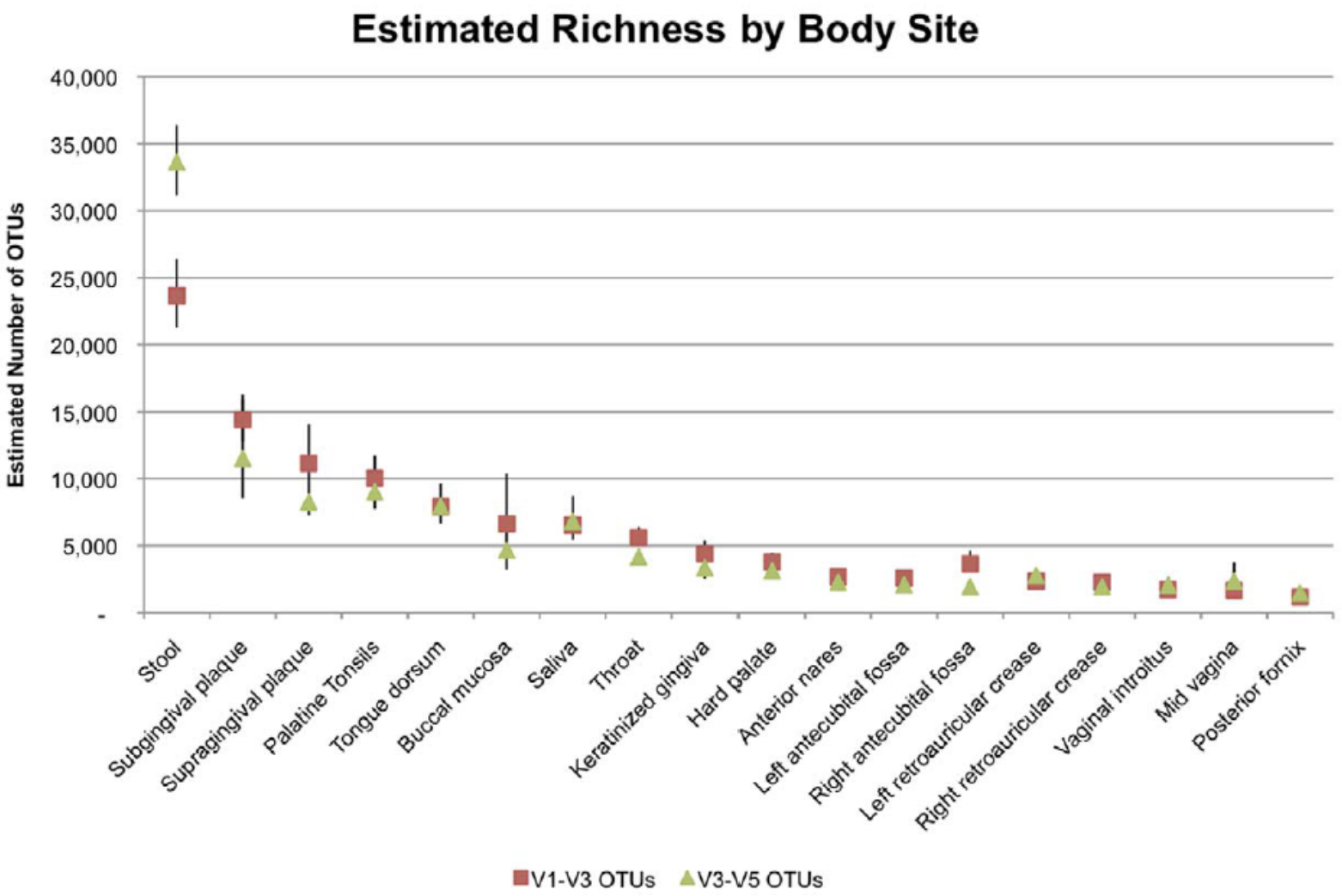
A Core Human Microbiome as Viewed through 16S rRNA Sequence Clusters

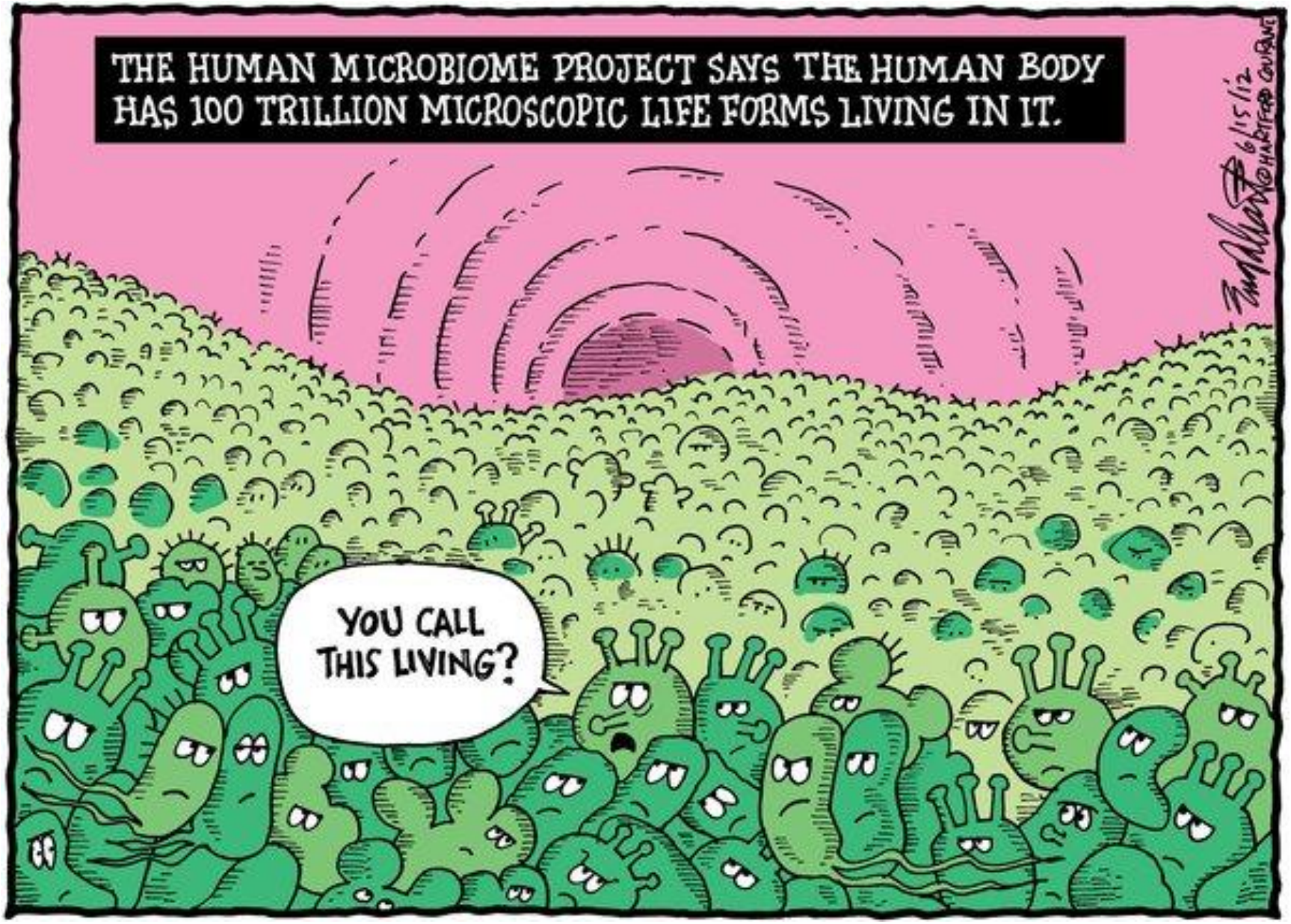
PLoS one

June 2012 | Volume 7 | Issue 6

Susan M. Huse[1]*, Yuzhen Ye[2], Yanjiao Zhou[3], Anthony A. Fodor[4]

Operational taxonomic units (OTUs)

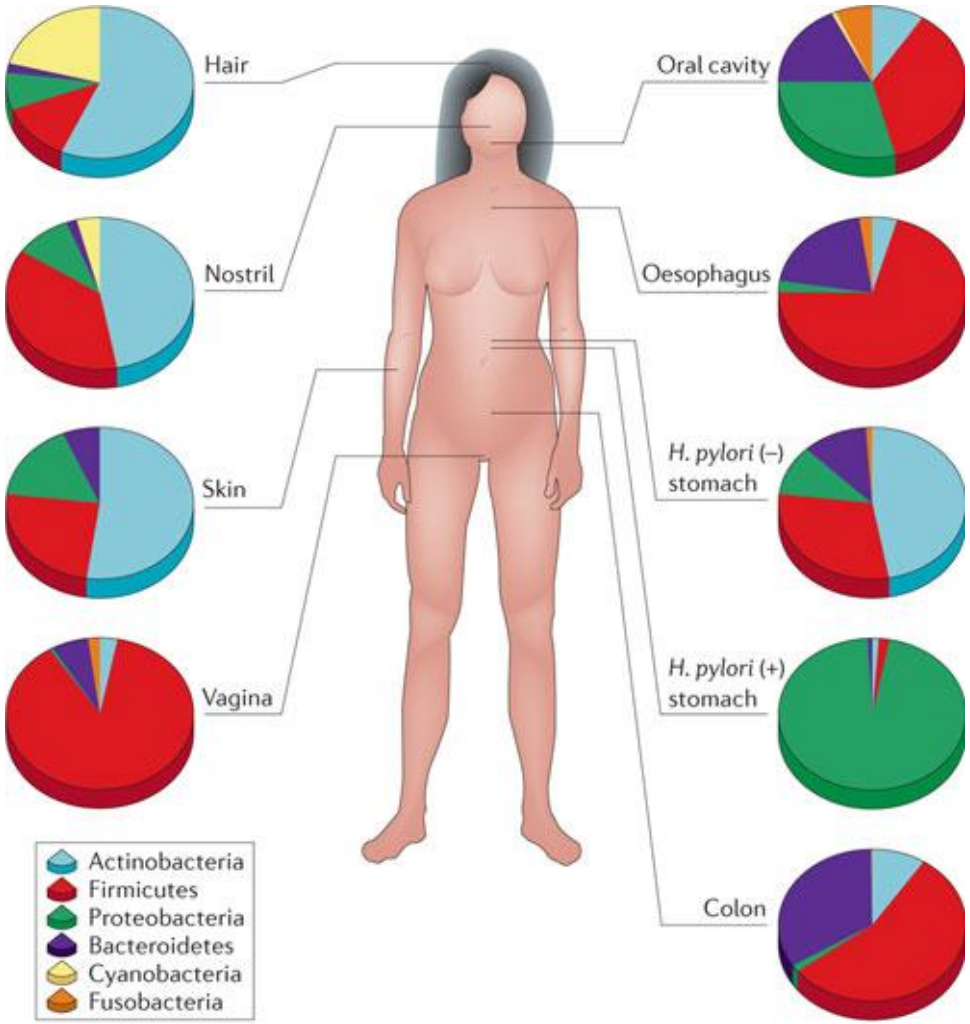




http://wenchwisdom.blogspot.com/2012/06/this-just-in-and-awesome-tending-human.html

The human microbiome: at the interface of health and disease

Ilseung Cho[1,2] *and Martin J. Blaser*[1,2,3,4]



Cho & Blaser, Nature Reviews Genetics 2012

Pakistan

Why might some kids have less bacteria than others?

http://wondersofpakistan.wordpress.com

Sweden

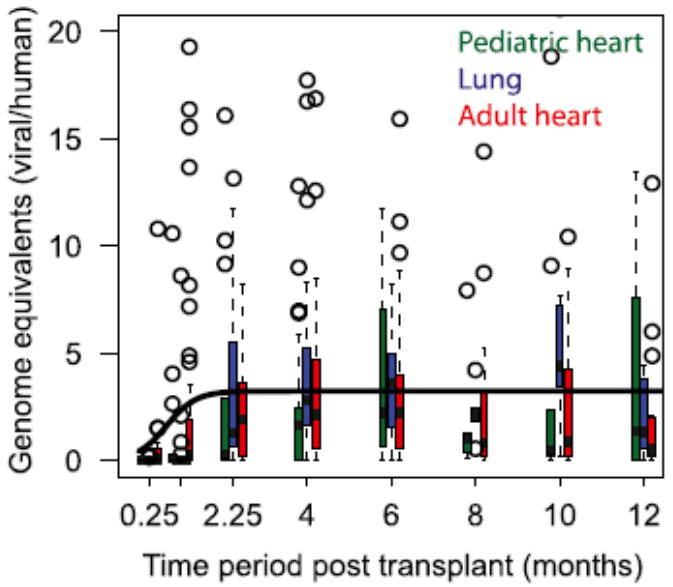
http://yourlivingcity.com/

Temporal Response of the Human Virome to Immunosuppression and Antiviral Therapy

Cell *155*, 1178–1187, November 21, 2013

Iwijn De Vlaminck,[1] Kiran K. Khush,[2] Calvin Strehl,[2] Bitika Kohli,[2] Helen Luikart,[2] Norma F. Neff,[1] Jennifer Okamot Thomas M. Snyder,[1] David N. Cornfield,[3] Mark R. Nicolls,[3] David Weill,[3] Daniel Bernstein,[4] Hannah A. Valantine,[2] and Stephen R. Quake[1,*]

Total viral load increases with immunosuppression



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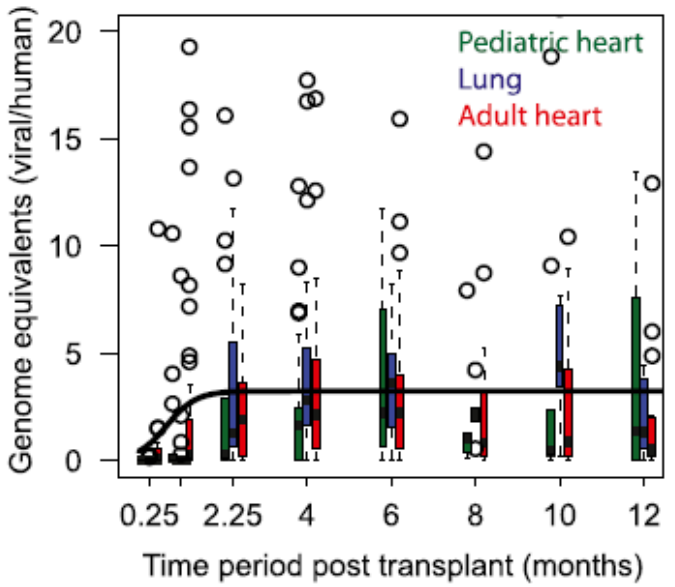
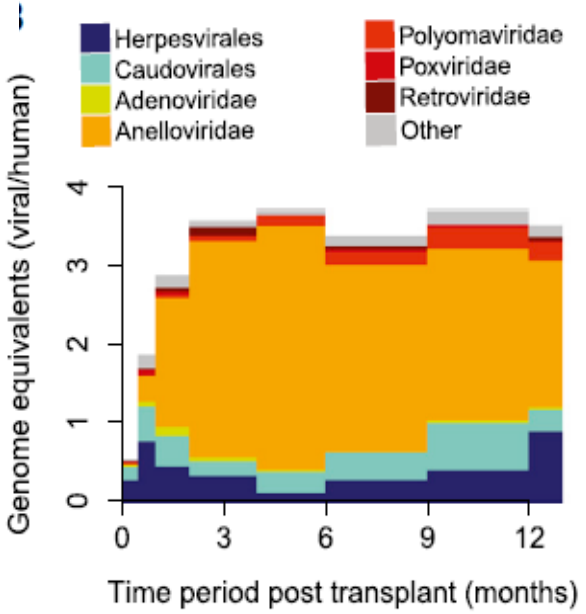
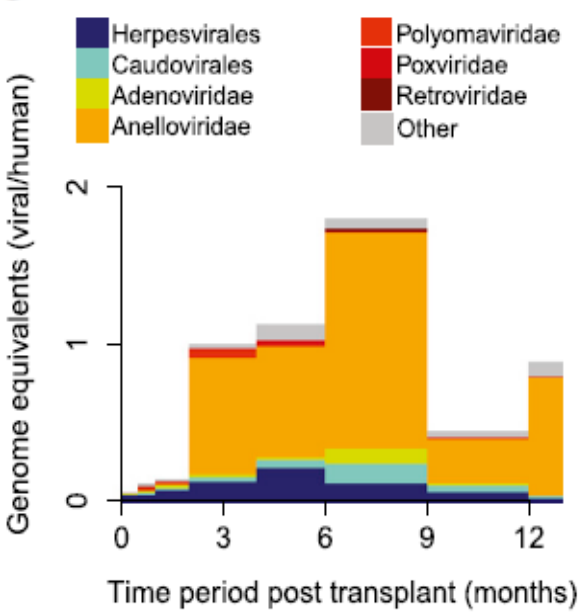


CMV+



CMV-



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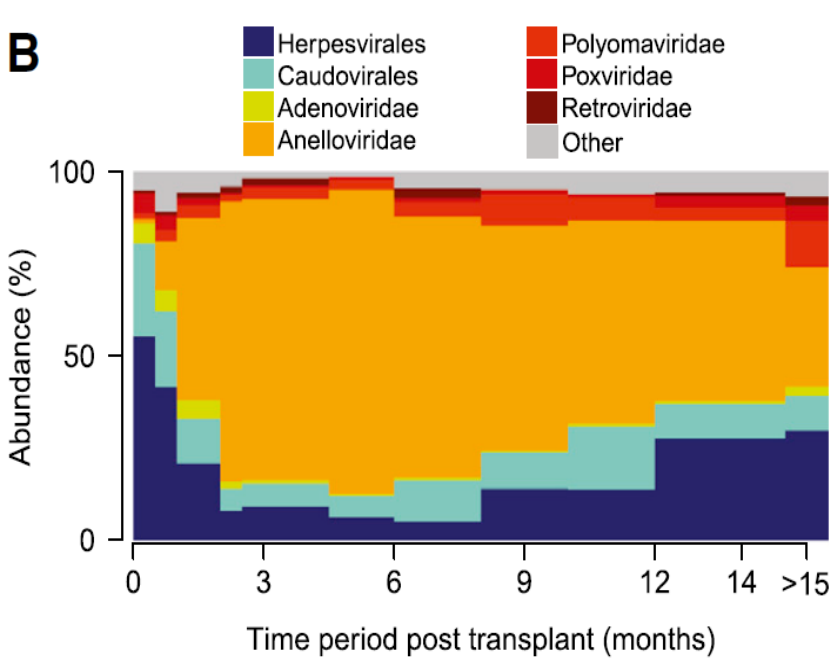
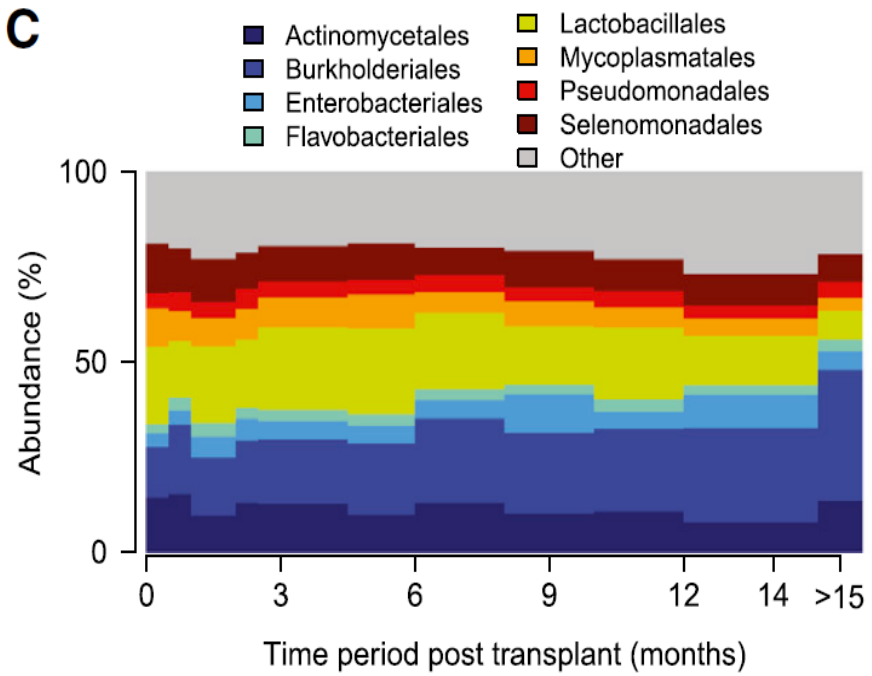
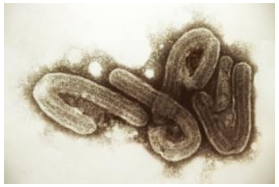
- **marked virome composition dynamics at the onset of the therapy**
- **total viral load increases with immunosuppression**
- **the bacterial component of the microbiome remains largely unaffected.**



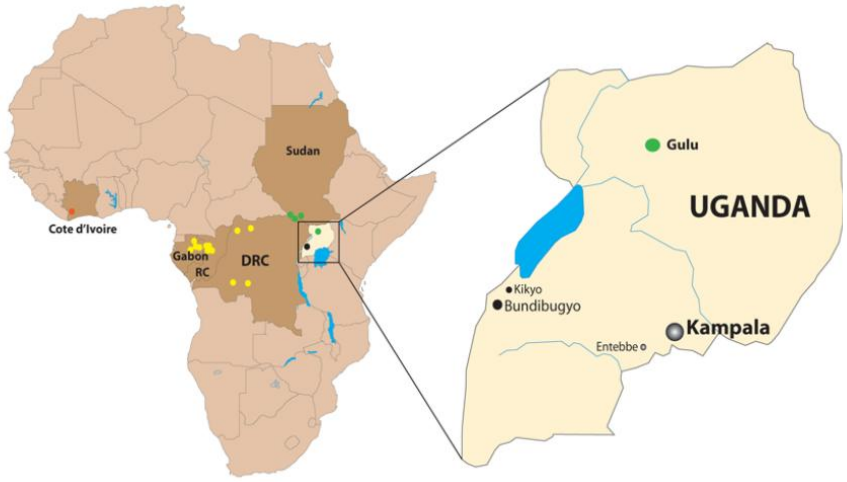


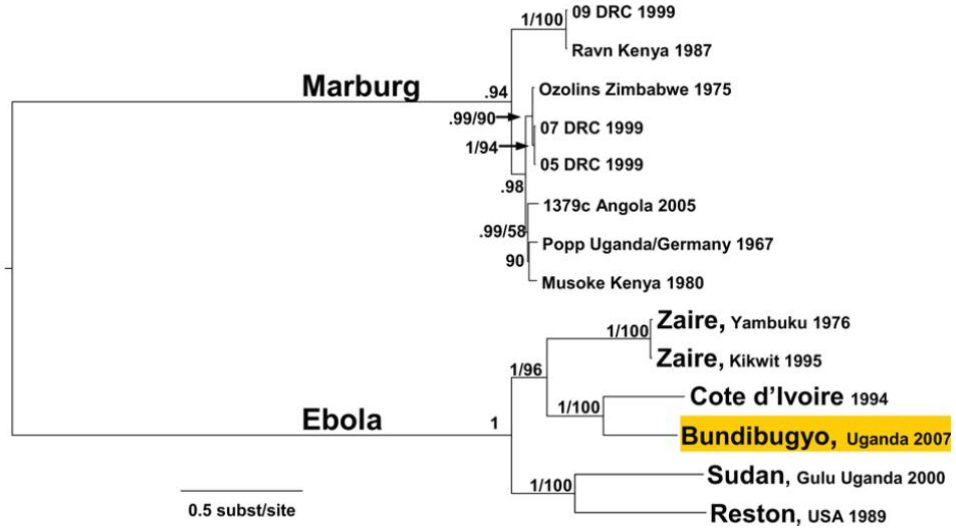
OPEN ACCESS Freely available online November 2008 | Volume 4 | Issue 11 | PLoS PATHOGENS

Newly Discovered Ebola Virus Associated with Hemorrhagic Fever Outbreak in Uganda



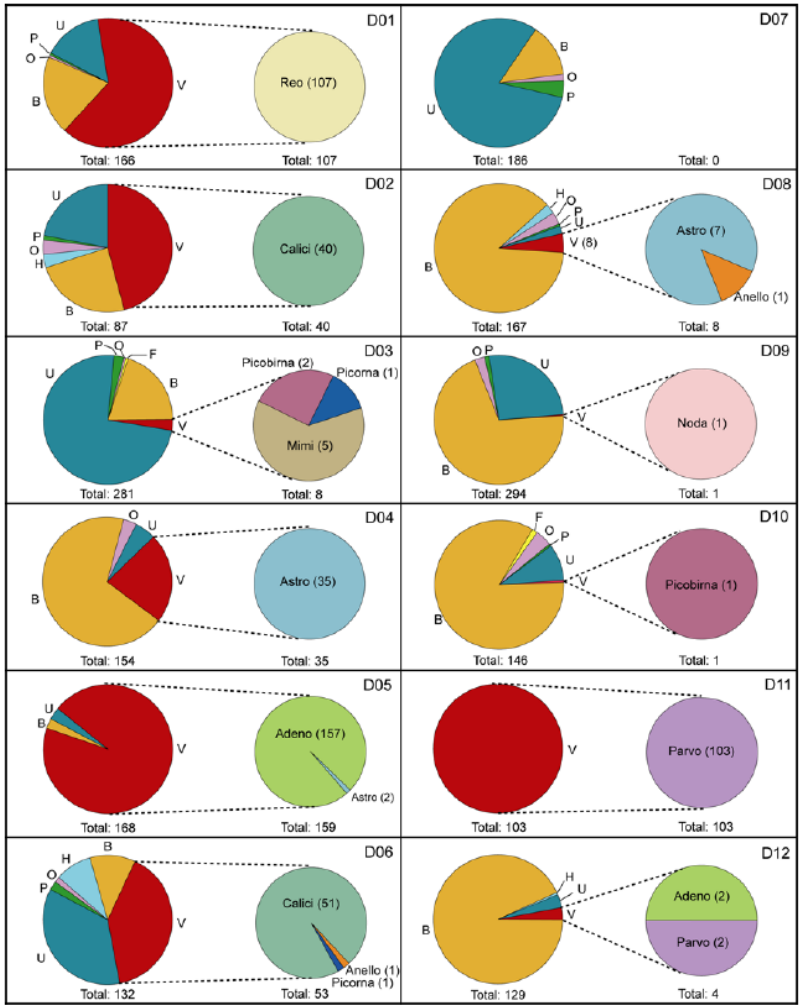
Jonathan S. Towner[1], Tara K. Sealy[1], Marina L. Khristova[2], César G. Albariño[1], Sean Conlan[3], Serena A. Reeder[1], Phenix-Lan Quan[3], W. Ian Lipkin[3], Robert Downing[4], Jordan W. Tappero[4], Samuel Okware[5], Julius Lutwama[6], Barnabas Bakamutumaho[6], John Kayiwa[6], James A. Comer[1], Pierre E. Rollin[1], Thomas G. Ksiazek[1], Stuart T. Nichol[1]*

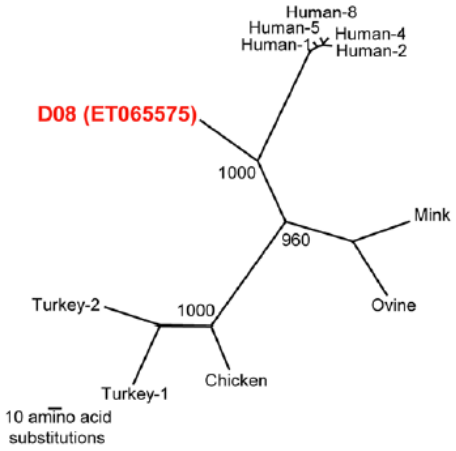


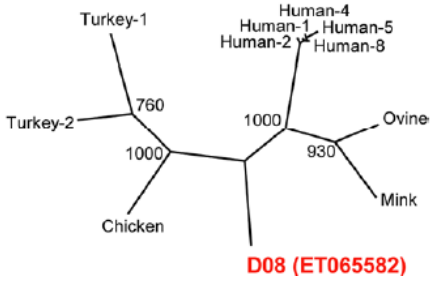


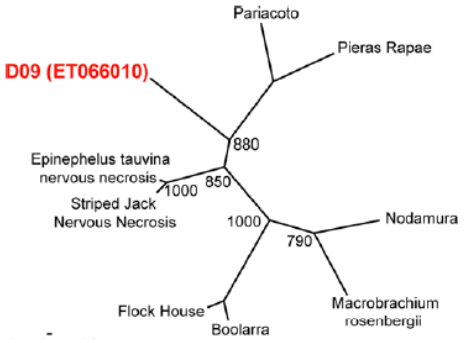
Metagenomic Analysis of Human Diarrhea: Viral Detection and Discovery

Stacy R. Finkbeiner[1,2☯], Adam F. Allred[1,2☯], Phillip I. Tarr[3], Eileen J. Klein[4], Carl D. Kirkwood[5], David Wang[1,2*]









Phylogenetic analysis of highly divergent astrovirus-like sequence reads and nodavirus-like sequence reads

PLoSpathogen, 2008

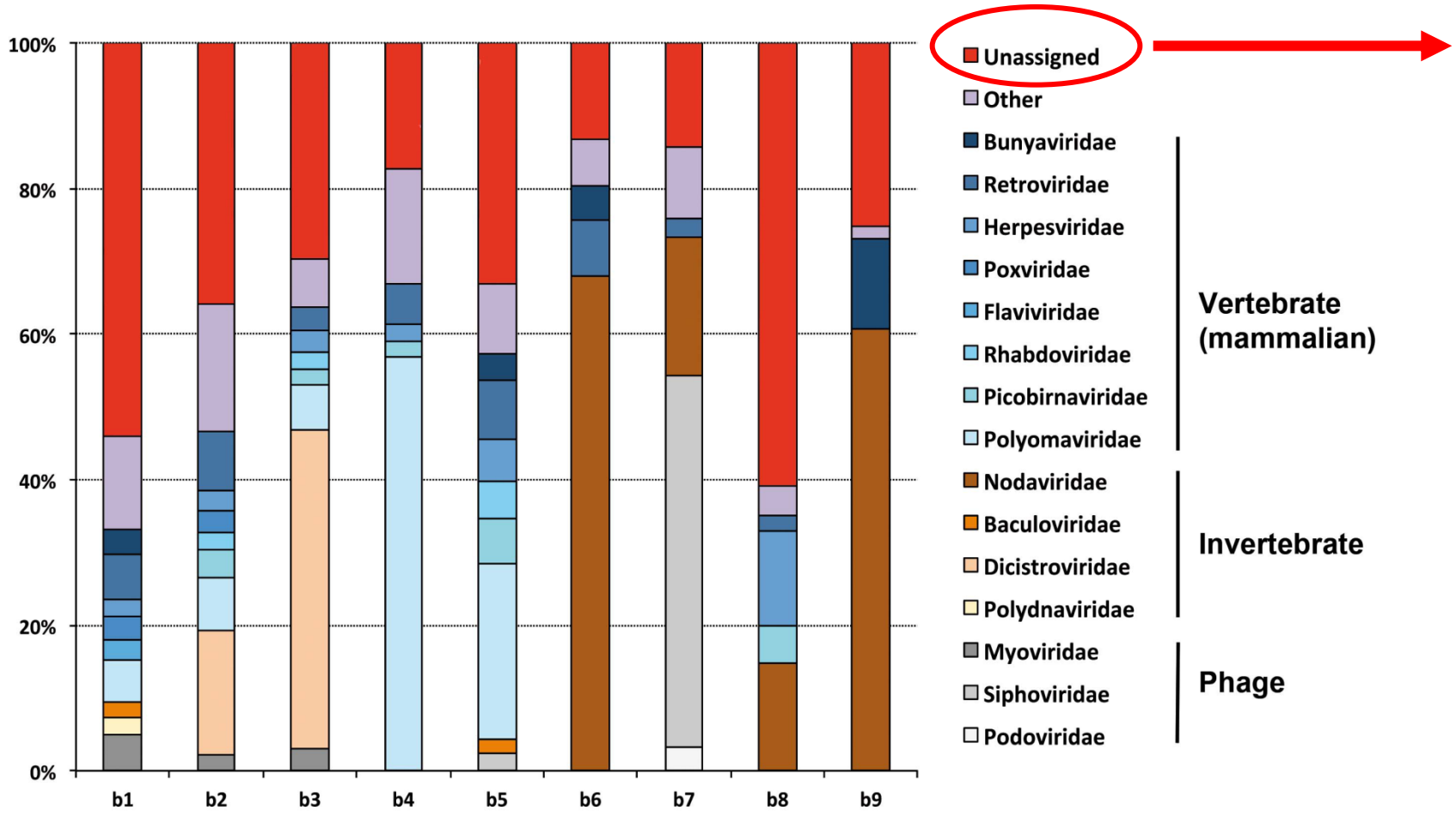
Bats: a Pandora's box for emerging viruses

A Preliminary Study of Viral Metagenomics of French Bat Species in Contact with Humans: Identification of New Mammalian Viruses

PLOS ONE | January 2014 | Volume 9 | Issue 1 | e87194

Laurent Dacheux[1]*, Minerva Cervantes-Gonzalez[1], Ghislaine Guigon[2], Jean-Michel Thiberge[2], Mathias Vandenbogaert[2], Corinne Maufrais[3], Valérie Caro[2☯], Hervé Bourhy[1☯]

Percentage of sequences related to the most abundant viral families



A Preliminary Study of Viral Metagenomics of French Bat Species in Contact with Humans: Identification of New Mammalian Viruses

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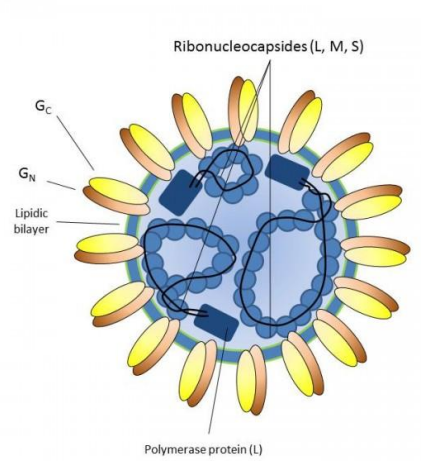
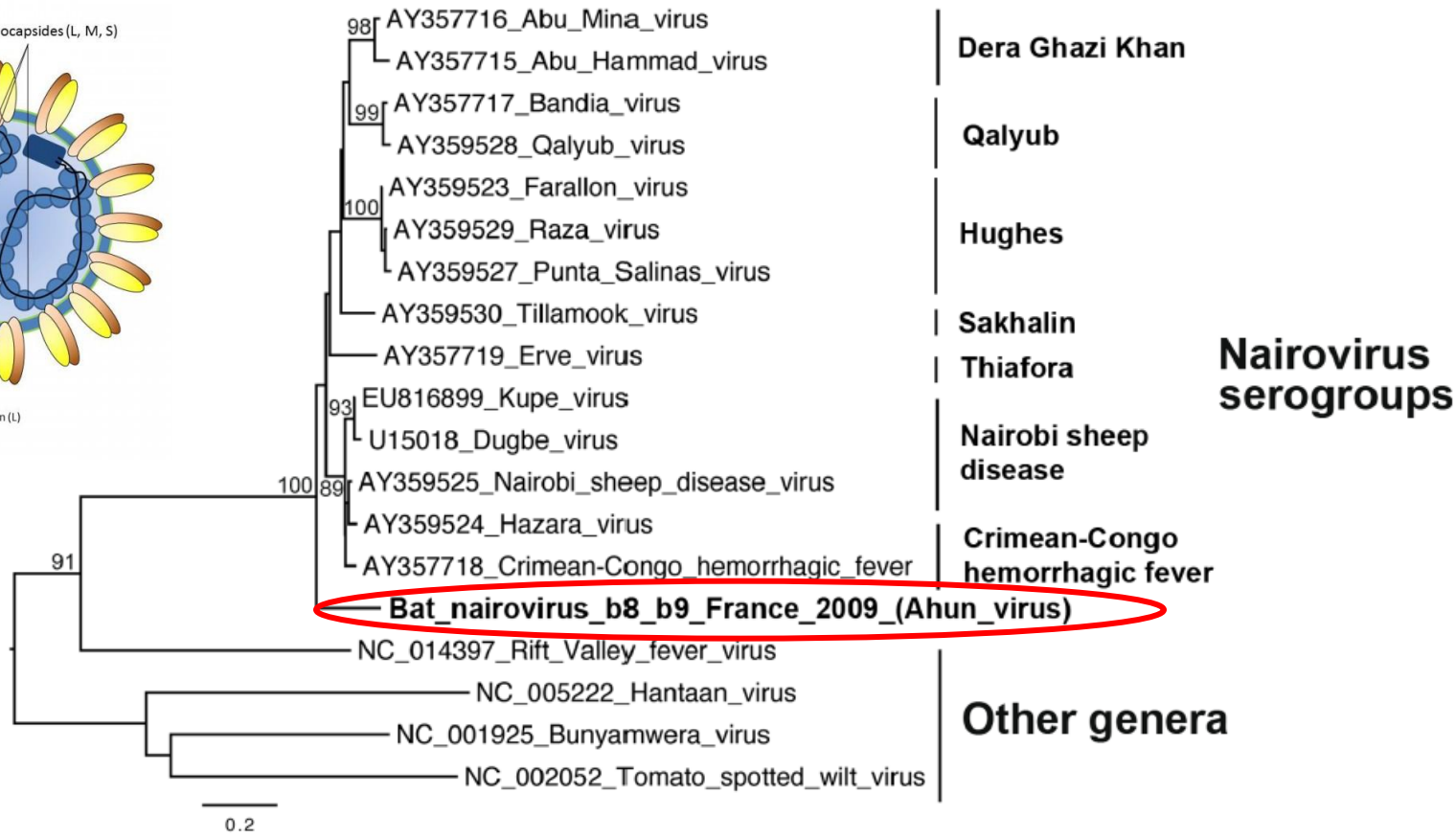
Among several new viruses identified, the first bat nairovirus





A Strategy To Estimate Unknown Viral Diversity in Mammals

mBio, 2013

Simon J. Anthony,[a,b] Jonathan H. Epstein,[b] Kris A. Murray,[b] Isamara Navarrete-Macias,[a] Carlos M. Zambrana-Torrelio,[b] Alexander Solovyov,[a] Rafael Ojeda-Flores,[c] Nicole C. Arrigo,[a] Ariful Islam,[b] Shahneaz Ali Khan,[d] Parviez Hosseini,[b] Tiffany L. Bogich,[e,f] Kevin J. Olival,[b] Maria D. Sanchez-Leon,[a,b] William B. Karesh,[b] Tracey Goldstein,[g] Stephen P. Luby,[h] Stephen S. Morse,[g,i] Jonna A. K. Mazet,[g] Peter Daszak,[b] W. Ian Lipkin[a]

…there are a minimum of 320,000 mammalian viruses awaiting discovery...

Presentation outline

- Different NGS approaches
  - ✓Unbiased (shotgun)
  - ✓AmpliSeq
- Metagenomics
- Viral quasispecies and minority genomes
  - ✓HIV tropism
  - ✓Implications for resistance
    - HIV
    - HBV
    - HCV

OPEN ACCESS Freely available online

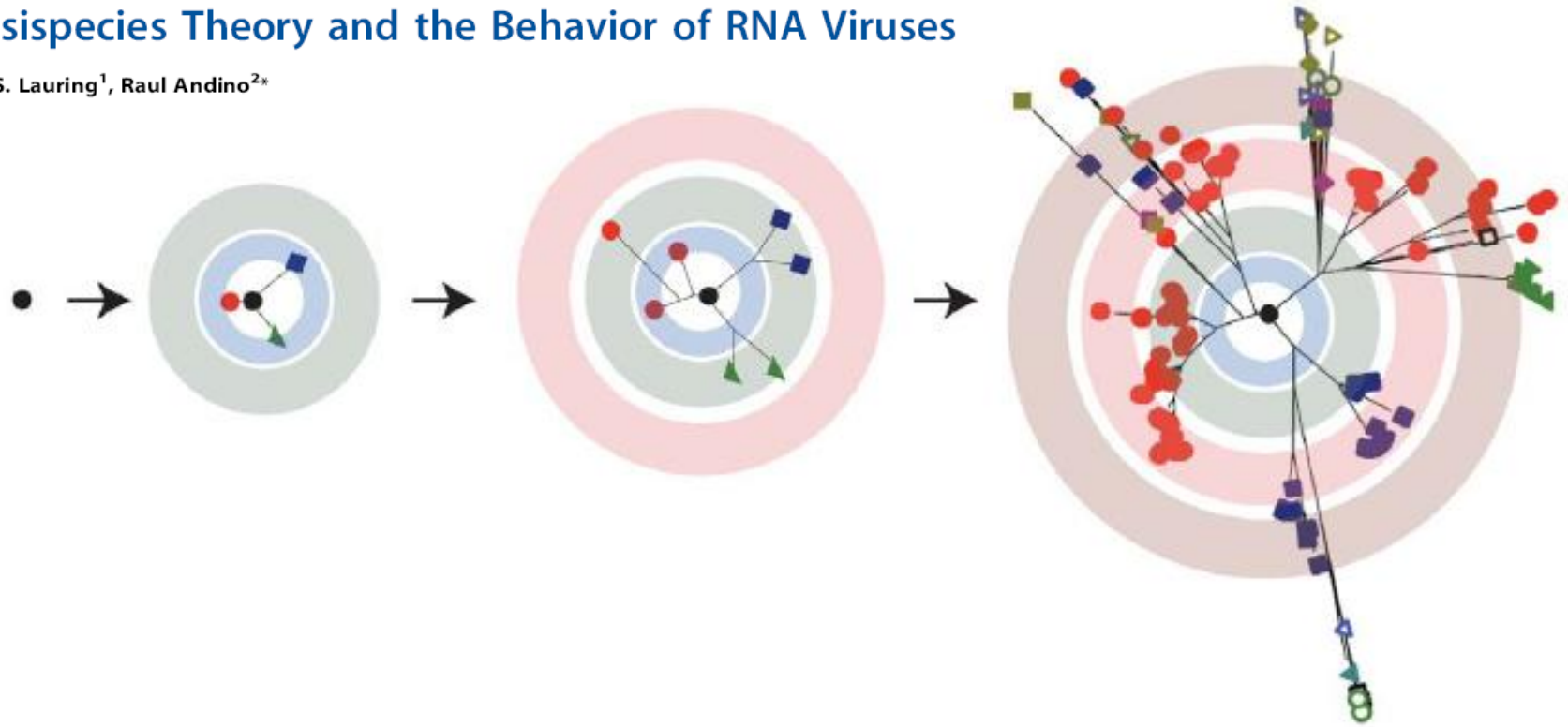
PLoS PATHOGENS

Review

July 2010 | Volume 6 | Issue 7 | e1001005

Quasispecies Theory and the Behavior of RNA Viruses

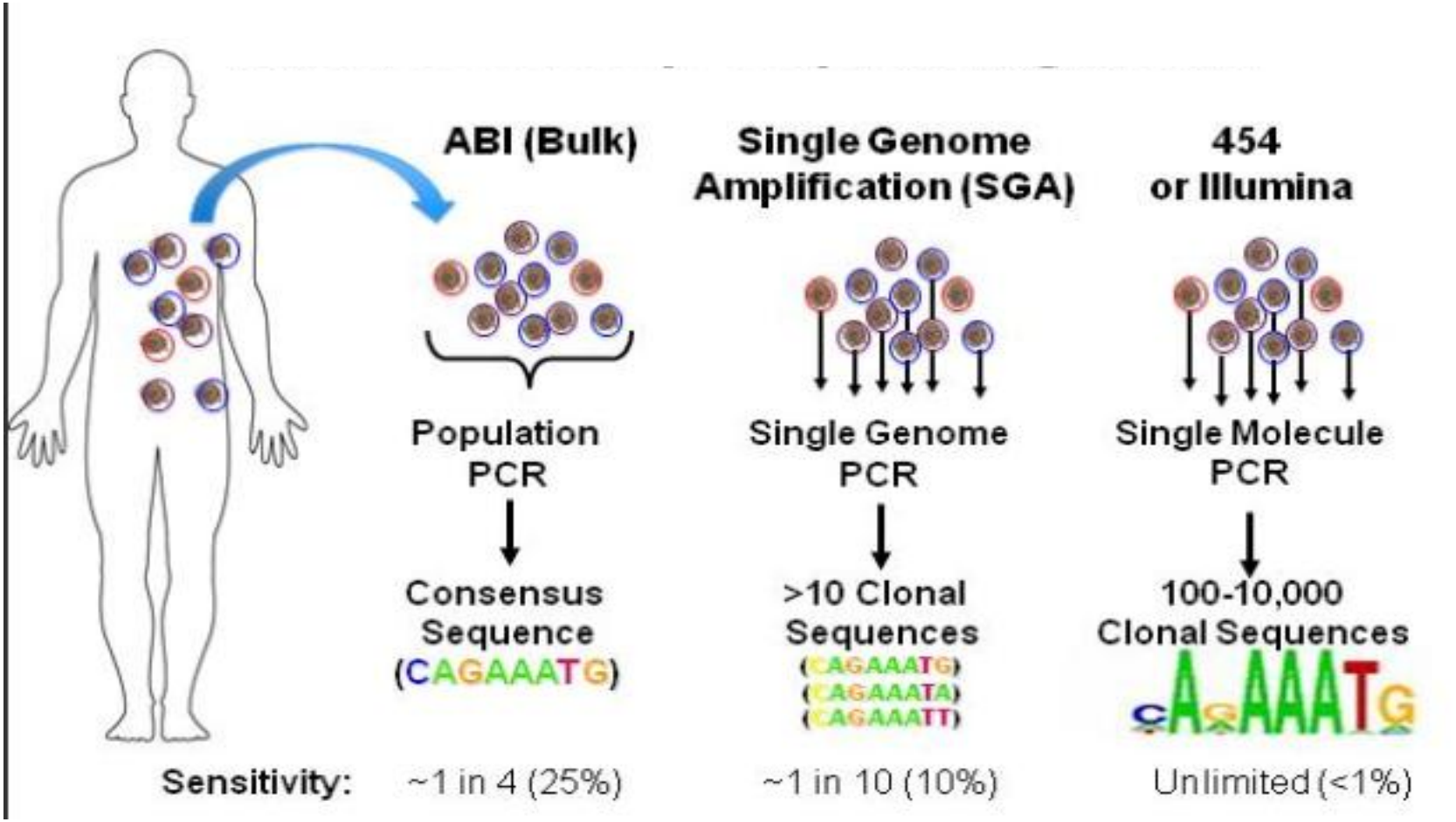
Adam S. Lauring[1], Raul Andino[2]*

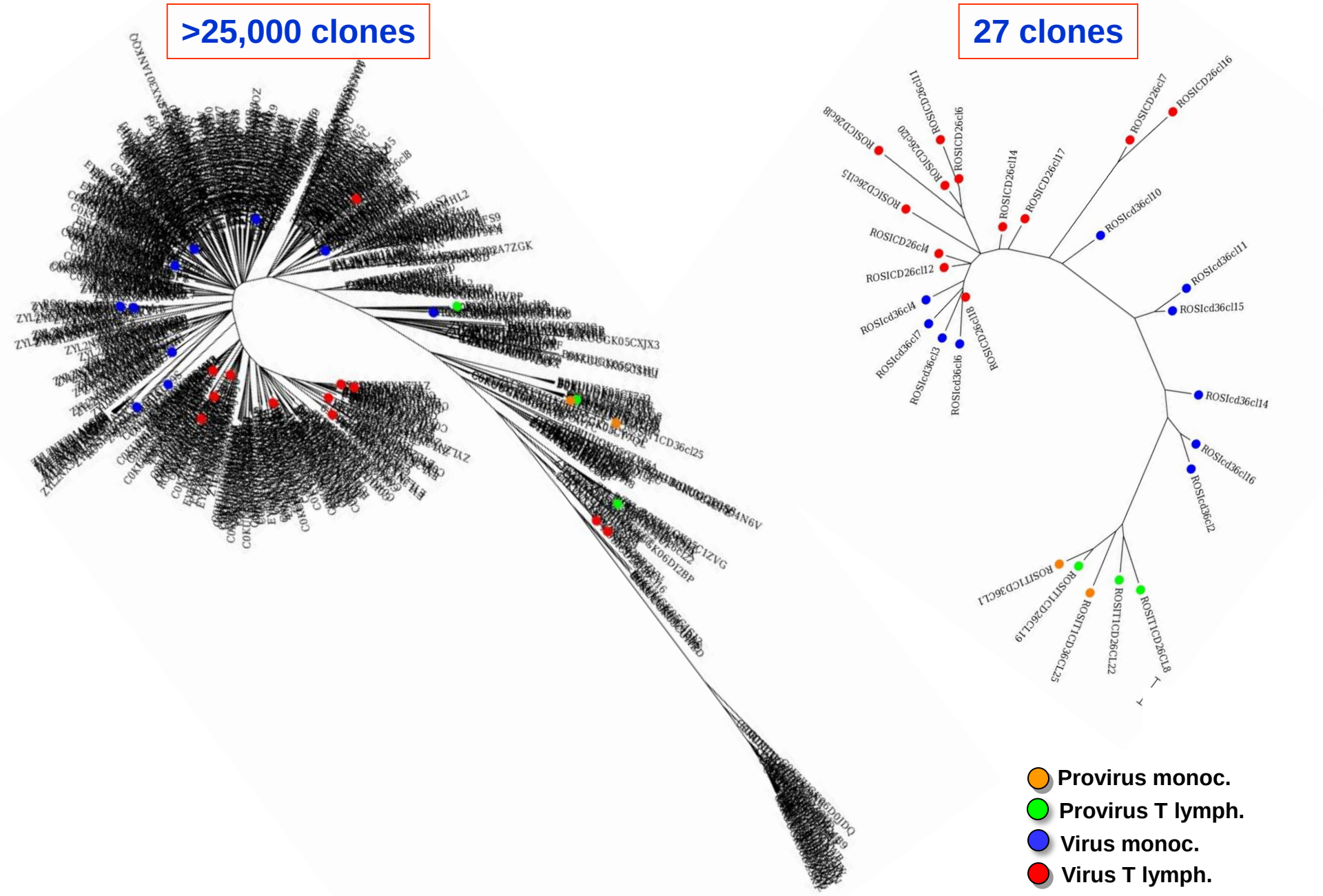


RNA viruses: error prone nature of the RNA-dependent RNA polymerase, 7×10^{-4} to 5.4×10^{-3} mutations per site per infectious cycle

Hepadnaviruses (RT-dependent): mutation rate 100-1000 times lower than RNA viruses, but about 100 x than other DNA viruses

Sequencing approaches to the study of quasispecies



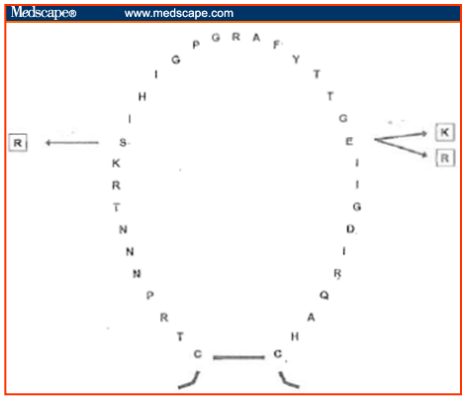

>25,000 clones
27 clones
ROSICD26cl11
ROSICD26cl20
ROSICD26cl6
ROSICD26cl16
ROSICD26cl7
ROSICD26cl8
ROSICD26cl14
ROSICD26cl17
ROSICD26cl15
ROSICD26cl10
ROSICD26cl4
ROSICD26cl12
ROSIcd36cl11
ROSIcd36cl15
ROSIcd36cl4
ROSIcd36cl7
ROSIcd36cl3
ROSIcd36cl6
ROSICD26cl18
ROSIcd36cl14
ROSIcd36cl16
ROSIcd36cl2
ROSITICD36CL1
ROSITICD26CL19
ROSITICD36CL25
ROSITICD26CL22
ROSITICD26CL18
Provirus monoc.
Provirus T lymph.
Virus monoc.
Virus T lymph.

Presentation outline

- Different NGS approaches
  - ✓Unbiased (shotgun)
  - ✓AmpliSeq
- Metagenomics
- Viral quasispecies and minority genomes
  - ✓HIV tropism
  - ✓Implications for resistance
    - HIV
    - HBV
    - HCV

Co-receptor usage predicted by Position Specific Score Matrices (PPSM) of variants present in viral quasispecies

V3 sequence is the main determinant of HIV tropism



Web PSSM

Enter your Email address to receive result via Email (Optional):

Enter your FASTA-formatted sequences **(amino acid sequences of the V3 loop of envelope gene)**:

Or upload sequence fasta file **(V3 loop amino acid sequences)**: Sfoglia...

Choose your matrix:
subtype B: x4r5 sinsi
subtype C: sinsi

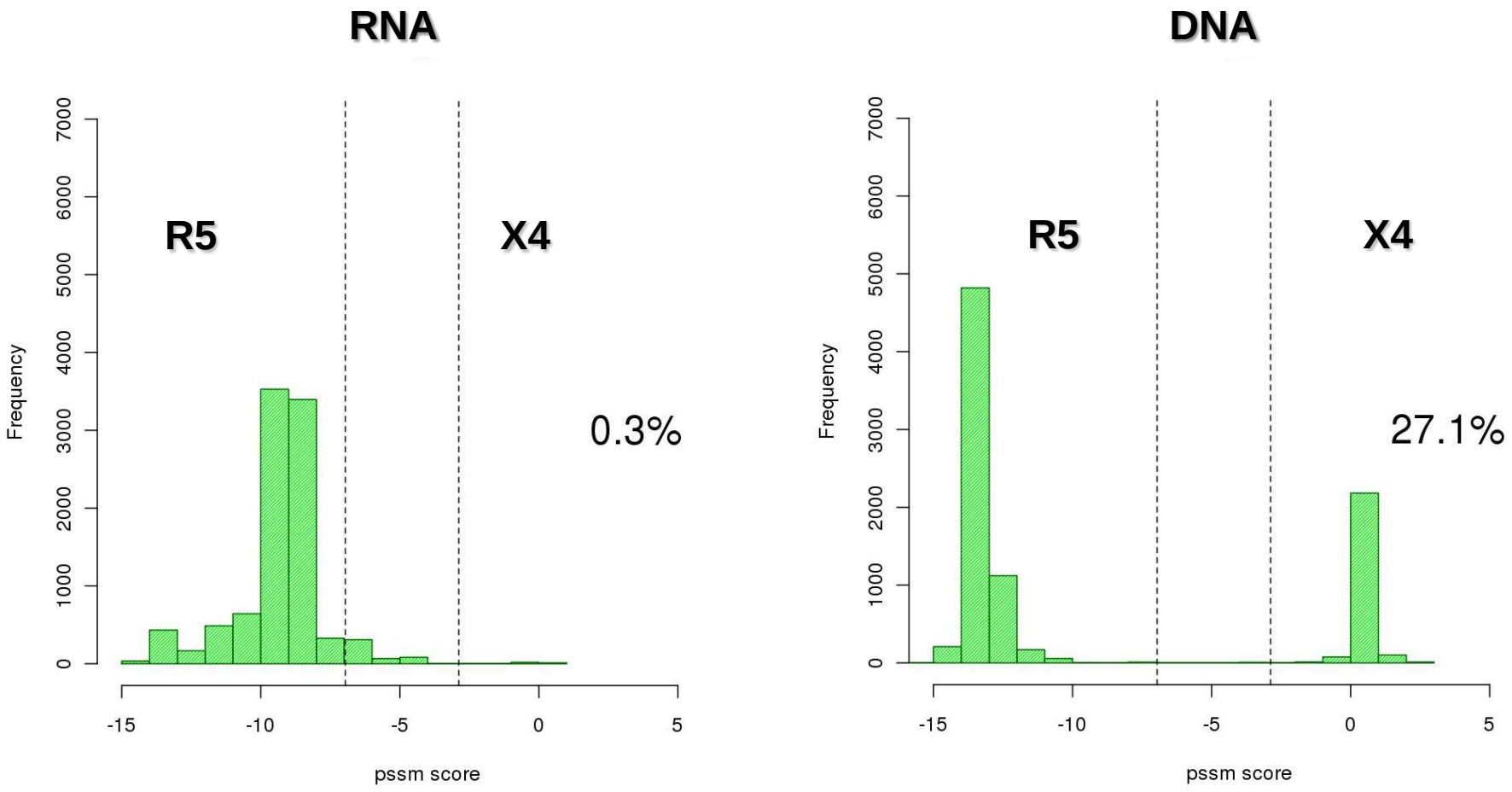
Reimposta DoPSSM

```
CTR-PNNNTR -KSISL---G PGRAFYATGD IVGNIRQAHC
CTR-PNNNTR -KSITL---G PGRAFYTTGQ IIGDIRKAHC
CTR-PSNNTR -KSITL---G PGRAFYATGD IIGDIRQAHR
CTR-PSNNTR -KSITL---G PGRAFYATGD IIGDIRQAHC
CTR-PSNNTR -KSINI---G PGSAWYATGD IIGDIRQAHC
CTR-PNSNTR -KSITL---G PGRAFYATGD IIGNIRQAHC
CTR-PSNNTR -KSIRI---G PGSAFYATGD IIGDIRQAHC
CTR-PSNNTR -KSISI---G PGSAWYATGD IIGDIRQAHC
CTR-PNNNTR -KSIRIQR-G PGRAFVTIGK I-GNMRQAHC
CTR-PNNNTR -KSITL---G PGRAFYATGN IIGNIRQAHC
CTR-PNNNTR -KSITL---G PGRAFYATGD VIGNIRQAHC
CTR-PNNNTR -KSITL---G PGKVYYTTGQ IVGDIRQAHC
CTR-PNNNTR -KSITL---G PGRAFYATGD IIGDIRQAHC
CTR-PNNNTM -KSITL---G PGRAFYTTGQ IIGDIRQAHC
RTR-PNNNTR -KSITL---G PGRAFYATGD IIGNIRQAHC
...
```

score	pred	geno	pos.chg	net.chg	reads	X4%
-10,48	0	SD	6	5	3	0,4
-8,03	0	SQ	7	6	3	0,4
-10,82	0	SD	7	5	2	0,27
-12,12	0	SD	6	4	110	14,82
-14,42	0	SD	5	3	34	4,58
-10,12	0	SD	6	5	2	0,27
-11,41	0	SD	6	4	19	2,56
-12,89	0	SD	5	3	10	1,35
0,71	1	SK	8	8	112	15,09
-8,89	0	SN	6	6	2	0,27
-10,28	0	SD	6	5	2	0,27
-5,32	0	SQ	6	5	3	0,4
-11,61	0	SD	6	4	3	0,4
-9,03	0	SQ	5	4	17	2,29
-9,49	0	SD	7	6	5	0,6
...						

1 predicts X4 or R5X4 phenotype and 0 predicts R5 phenotype

UDPS allows detailed description of quasispecies tropism in both plasma viral RNA and proviral DNA



Vertical lines (-2.88 and -6.96) : 95th and 5th percentiles for X4 and R5

Score of reference strains: R5 BaL = -12.96

X4 HXB2= +3.47

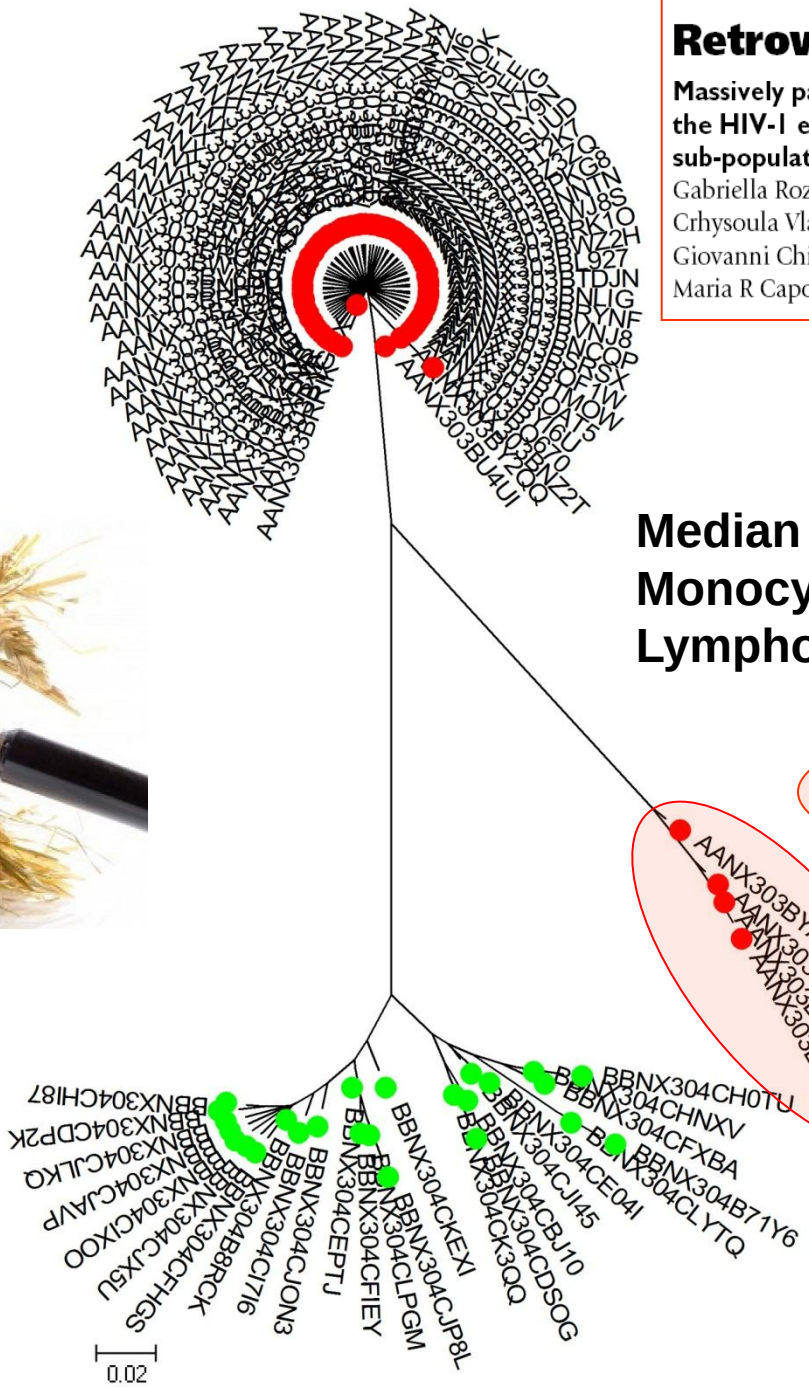
Abbate I. Clin Microb Infect 2010

Pt.4 after 2 years on HAART

Retrovirology
Published: 12 February 2009
Retrovirology 2009, 6:15 doi:10.1186/1742-4690-6-15
BioMed Central

Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations

Gabriella Rozera[1], Isabella Abbate*[1], Alessandro Bruselles[1], Crhysoula Vlassi[2], Gianpiero D'Offizi[2], Pasquale Narciso[2], Giovanni Chillemi[3], Mattia Prosperi[1], Giuseppe Ippolito[4] and Maria R Capobianchi[1]



Median score in
Monocytes: -11.60 (R5)
Lymphocytes: - 8.43 (R5)

Frequency: 4.2%
Score: +3.76 (X4)

Reference		Score
R5	BaL	-12.96
X4	HXB2	+3.47

Provirus CD36 T0
Provirus CD26 T0

HIV-1 RNA V3 quasispecies and co-receptor usage in acute patients according to need of early cART

cART	CD4 cells/ul	HIV RNA Log cp/ml	% pts ** with X4	X4 * Log cp/ml	Diversity* subst/site
Yes n=10	**323** (191-809)	**5.4** (4.0-7.9)	**60**	**3.65** (<2.7-3.50)	**0.063** (0.004-0.38)
Not n=10	**700** (451-1251)	**5.0** (4.0-6.0)	**40**	**2.70** (<2-3.50)	**0.007** (0.004-0.11)
p	0.002	NS	NS	0.009	0.019

*median (range)
**>0.3%

Abbate I. AIDS 2010

HIV-1 RNA V3 quasispecies and co-receptor usage in acute patients according to need of early cART

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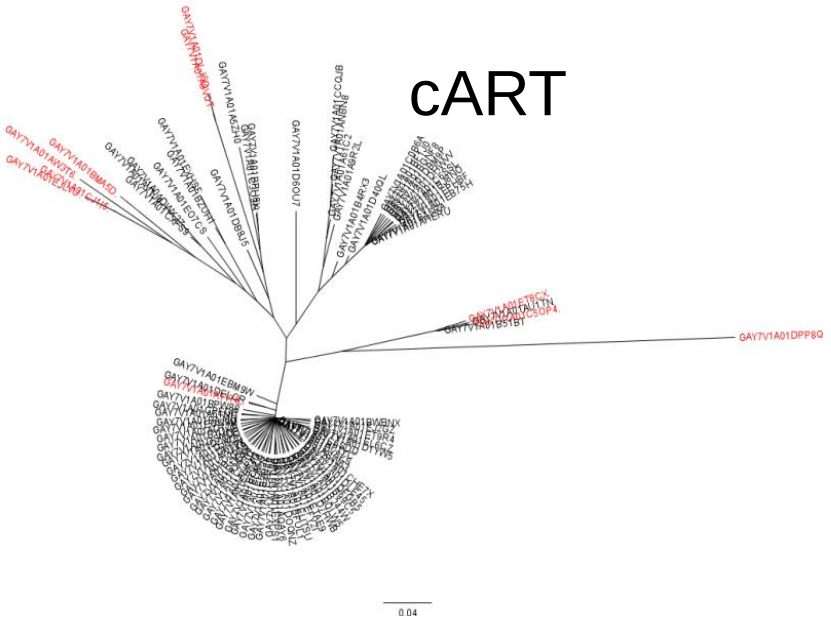
cART

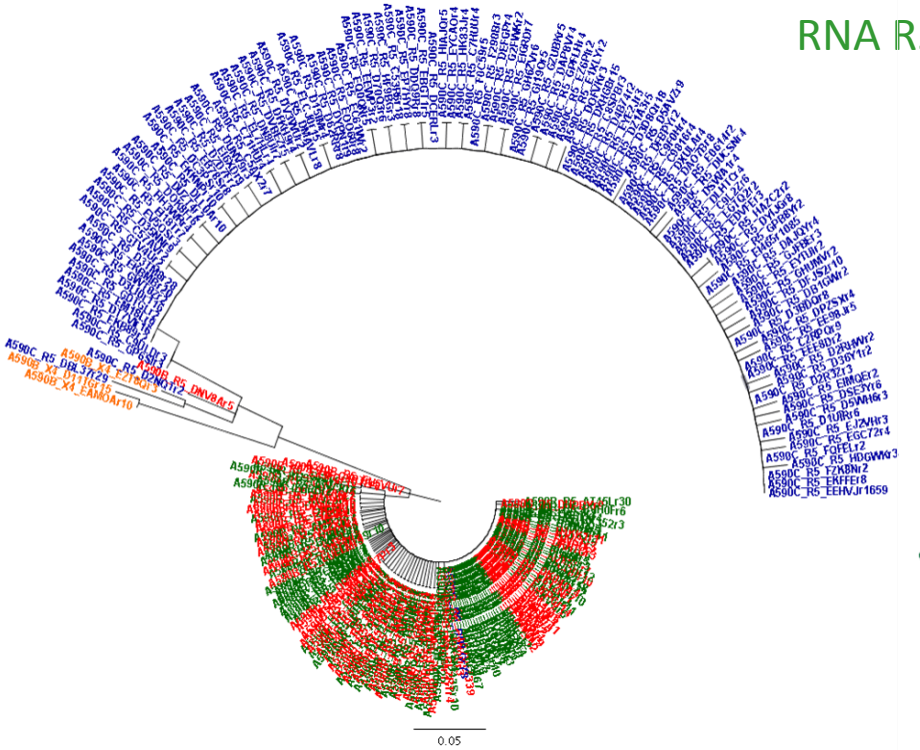


No cART

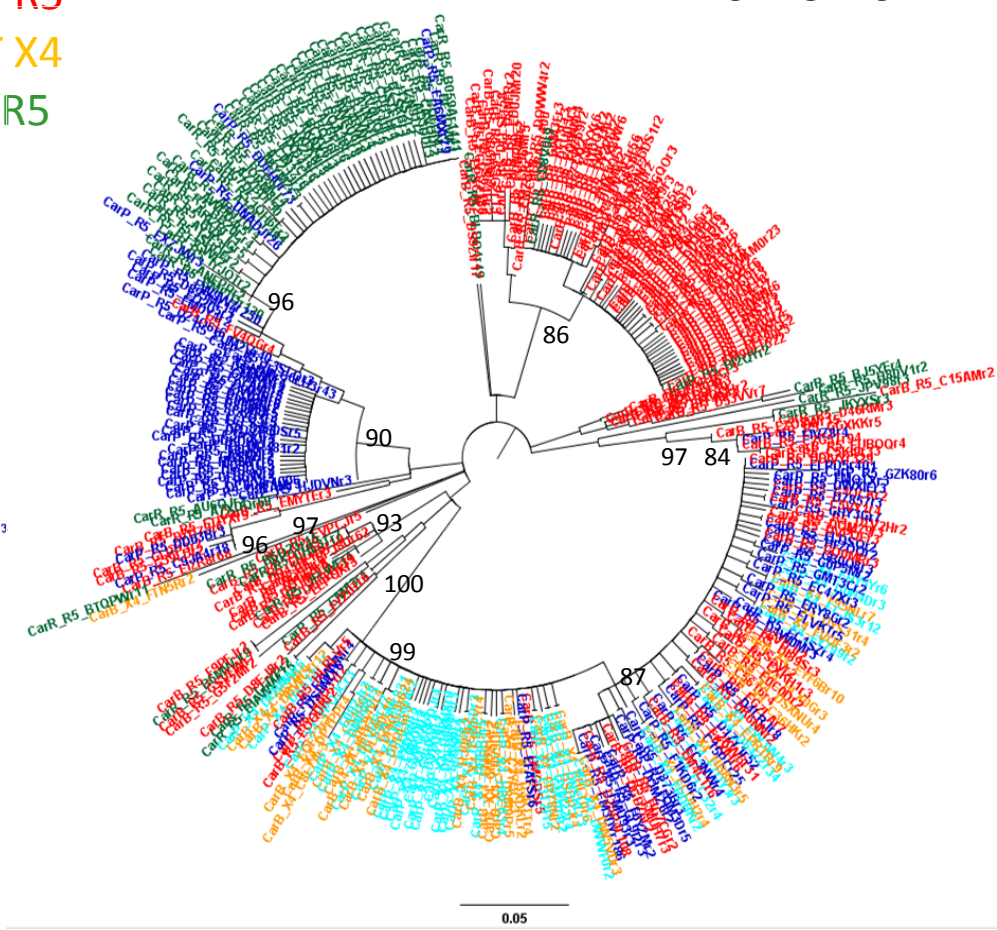
HIV compartmentalization during primary infection (V3 env)

PBMC R5
PBMC X4
GALT R5
GALT X4
RNA R5

primary



chronic



Bootstrap > 80

Rozera et al, Clin Microbiol Infect 2014

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  - ✓HIV tropism
  - ✓Implications for resistance
    - HIV
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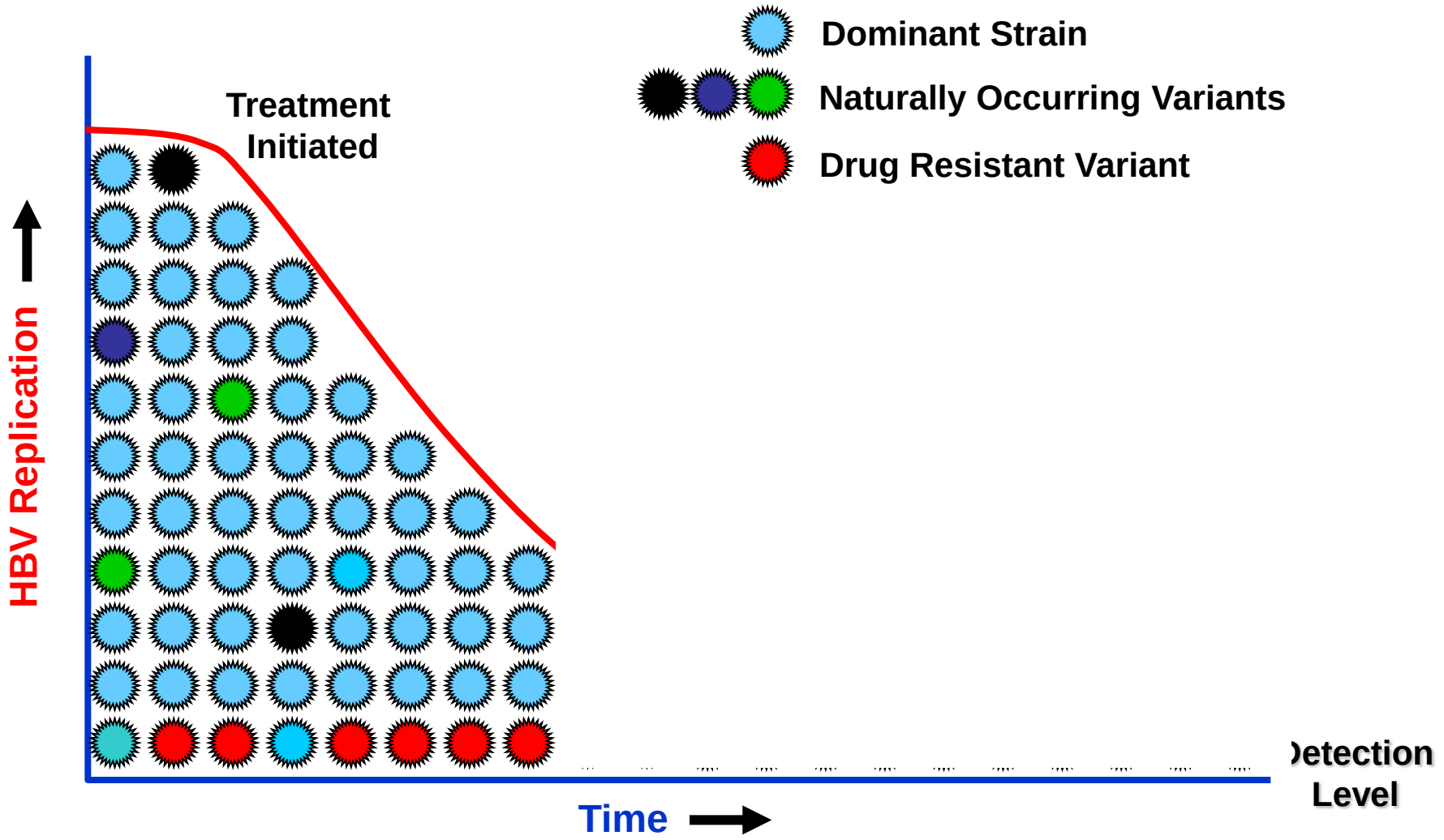
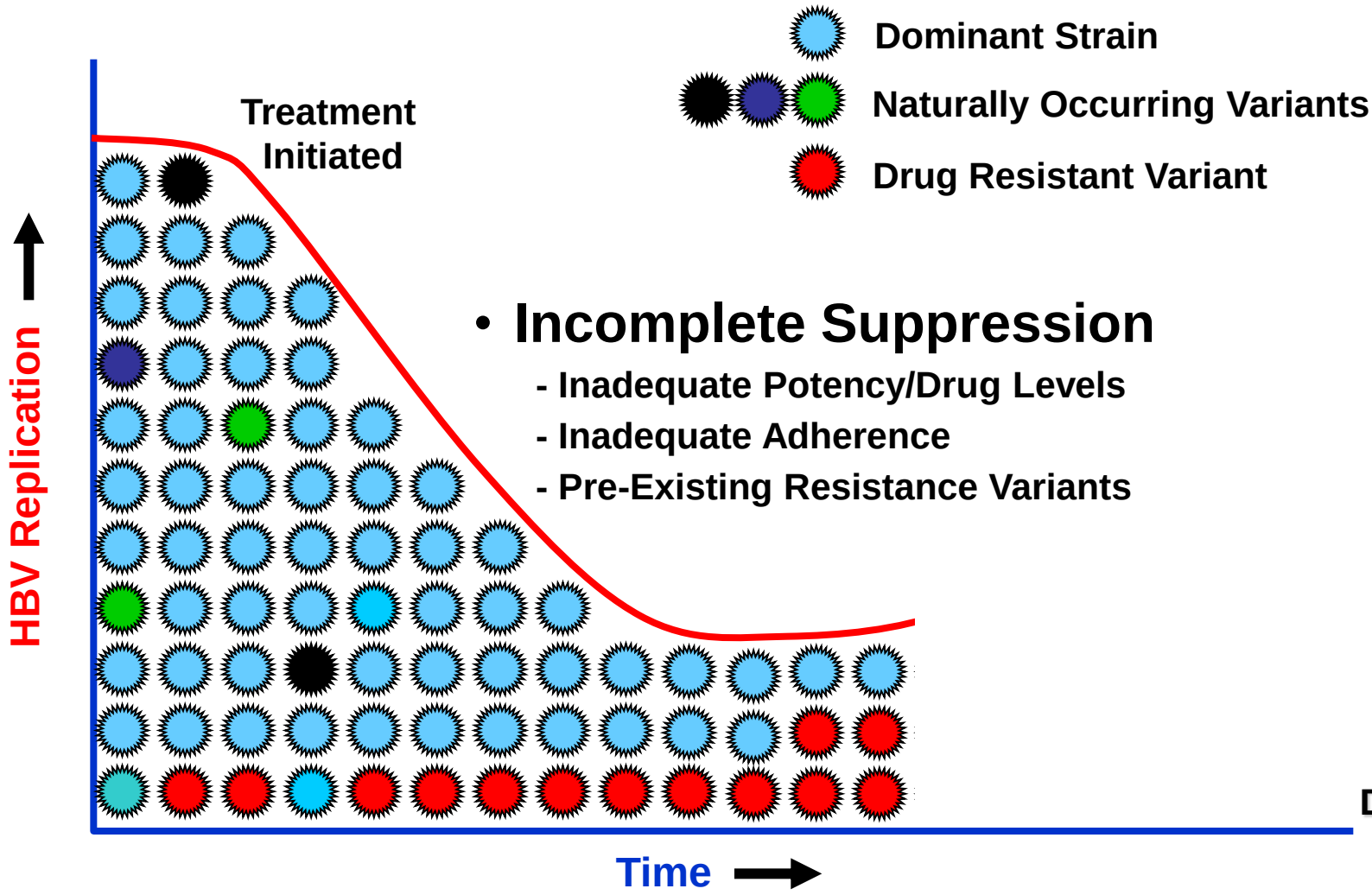
    - HCV

Incomplete suppression of virus replication leads to selection of mutants



Fung SK & Lok ASF. Antivir Ther 2004; 9:1013–1026
Locarnini S, et al. Antivir Ther 2004; 9:679–693

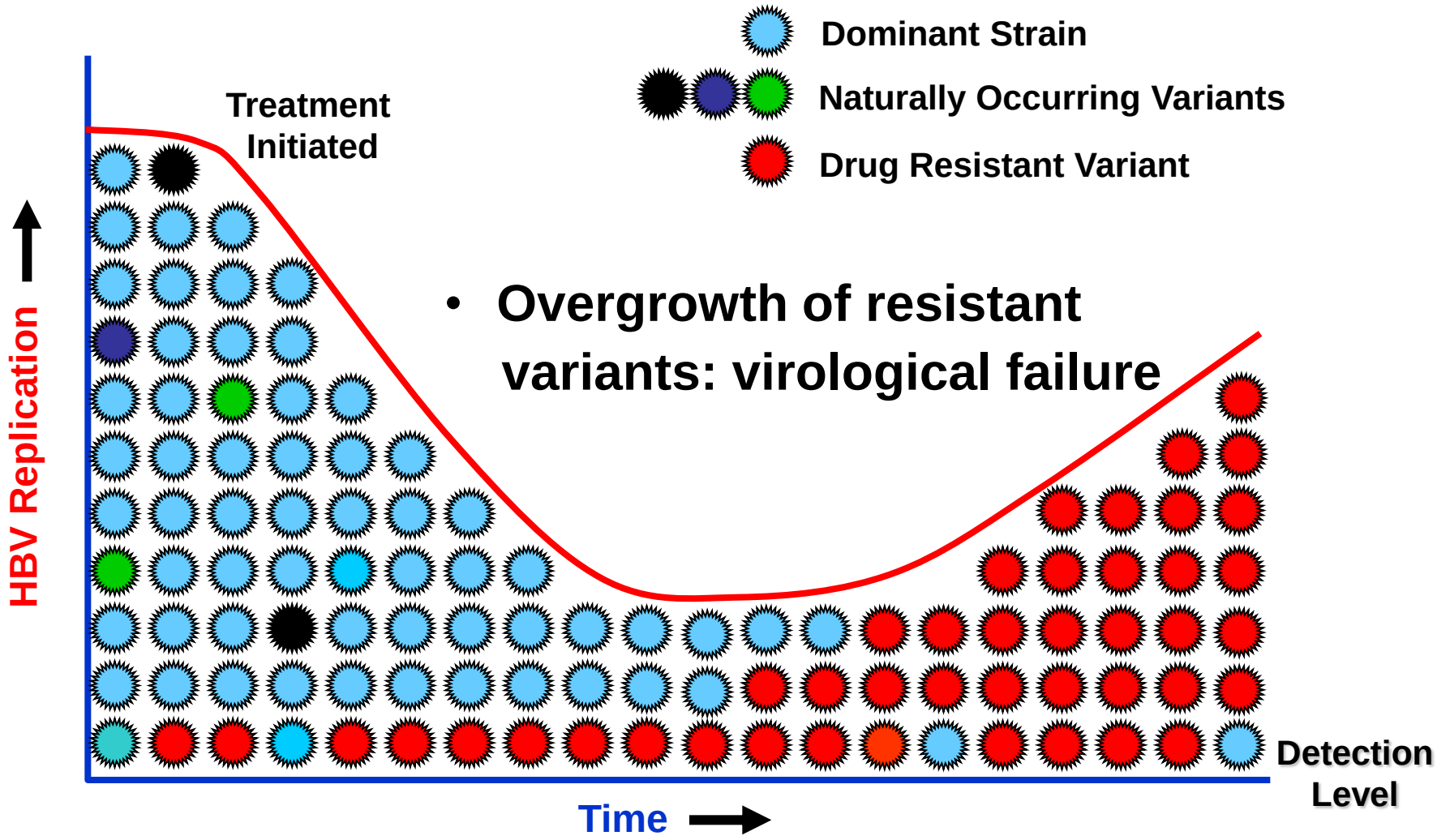
Incomplete suppression of virus replication leads to selection of mutants



- **Incomplete Suppression**
  - Inadequate Potency/Drug Levels
  - Inadequate Adherence
  - Pre-Existing Resistance Variants

Fung SK & Lok ASF. Antivir Ther 2004; 9:1013–1026
Locarnini S, et al. Antivir Ther 2004; 9:679–693

Incomplete suppression of virus replication leads to selection of mutants



Fung SK & Lok ASF. Antivir Ther 2004; 9:1013–1026
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JOURNAL OF VIROLOGY, Feb. 2009, p. 1718–1726 Vol. 83, No. 4
0022-538X/09/$08.00+0 doi:10.1128/JVI.02011-08

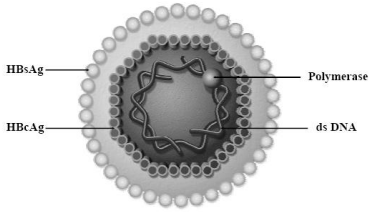
Use of Massively Parallel Ultradeep Pyrosequencing To Characterize the Genetic Diversity of Hepatitis B Virus in Drug-Resistant and Drug-Naive Patients and To Detect Minor Variants in Reverse Transcriptase and Hepatitis B S Antigen[▽]†

Mariacarmela Solmone, Donatella Vincenti, Mattia Carlo Felice Prosperi, Alessandro Bruselles, Giuseppe Ippolito, and Maria Rosaria Capobianchi*

National Institute for Infectious Diseases Lazzaro Spallanzani, Rome, Italy

NGS and HBV

Dynamics of HBV resistance mutations



HBV structural schematic

Direct sequecing : WT A181A/V, N236N/T

Pyrosequencing:

	Freq. %	Freq. %
L80I	2	<0.5
L180M	3.33	1.52
A181V	7.43	45.49
M204V	2.24	0.74
V214A	1.07	<0.5
N236T	12.03	63.2

stop lamivudine
start adefovir

start pegIFN

HBV DNA
UI/ml

1000000
100000
10000
1000
100
10
1

Sept. 2006

June 2006

Stop adefovir

LDL

apr-03 lug-03 ott-03 gen-04 apr-04 lug-04 ott-04 gen-05 apr-05 lug-05 ott-05 gen-06 apr-06 lug-06 ott-06 gen-07 apr-07 lug-07 ott-07

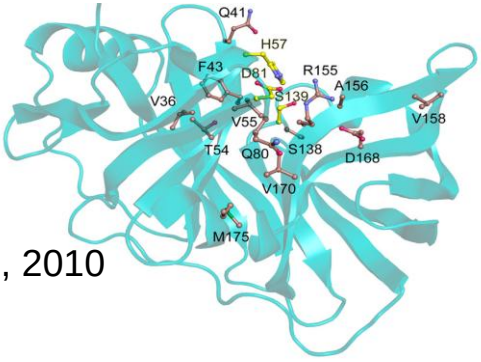
Menzo et al. JID 2009

Virus Research
Volume 177, Issue 2, 6 November 2013, Pages 205–208

Short communication

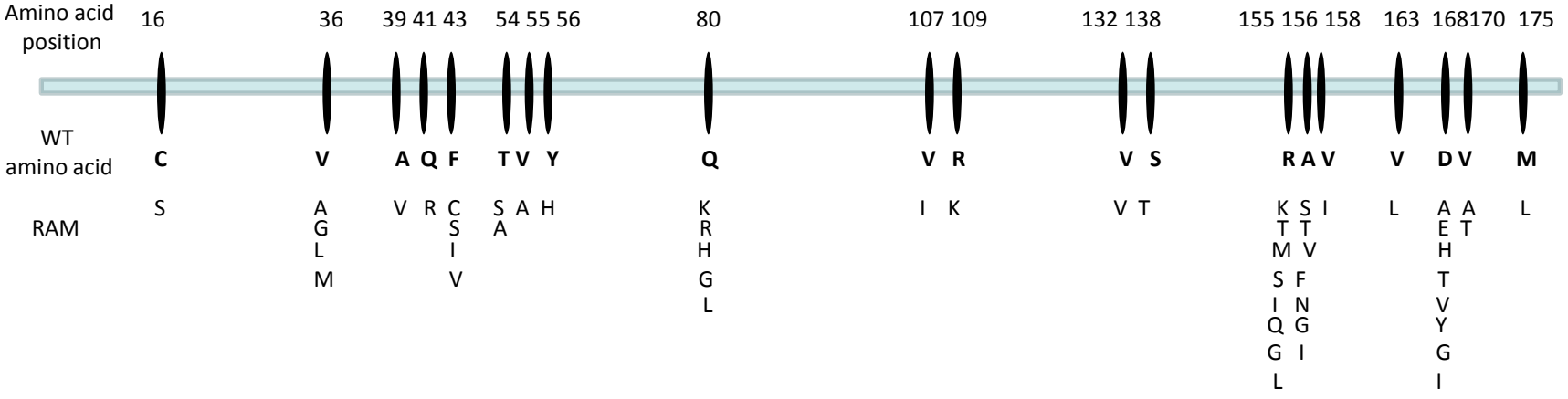
Extent of HCV NS3 protease variability and resistance-associated mutations assessed by next generation sequencing in HCV monoinfected and HIV/HCV coinfected patients

Barbara Bartolini, Emanuela Giombini, Paola Zaccaro, Marina Selleri, Gabriella Rozera, Isabella Abbate, Ubaldo Visco Comandini, Giuseppe Ippolito, Mariacarmela Solmone, Maria R. Capobianchi



Romano et al. PNAS, 2010

NS3 positions analysed in detail



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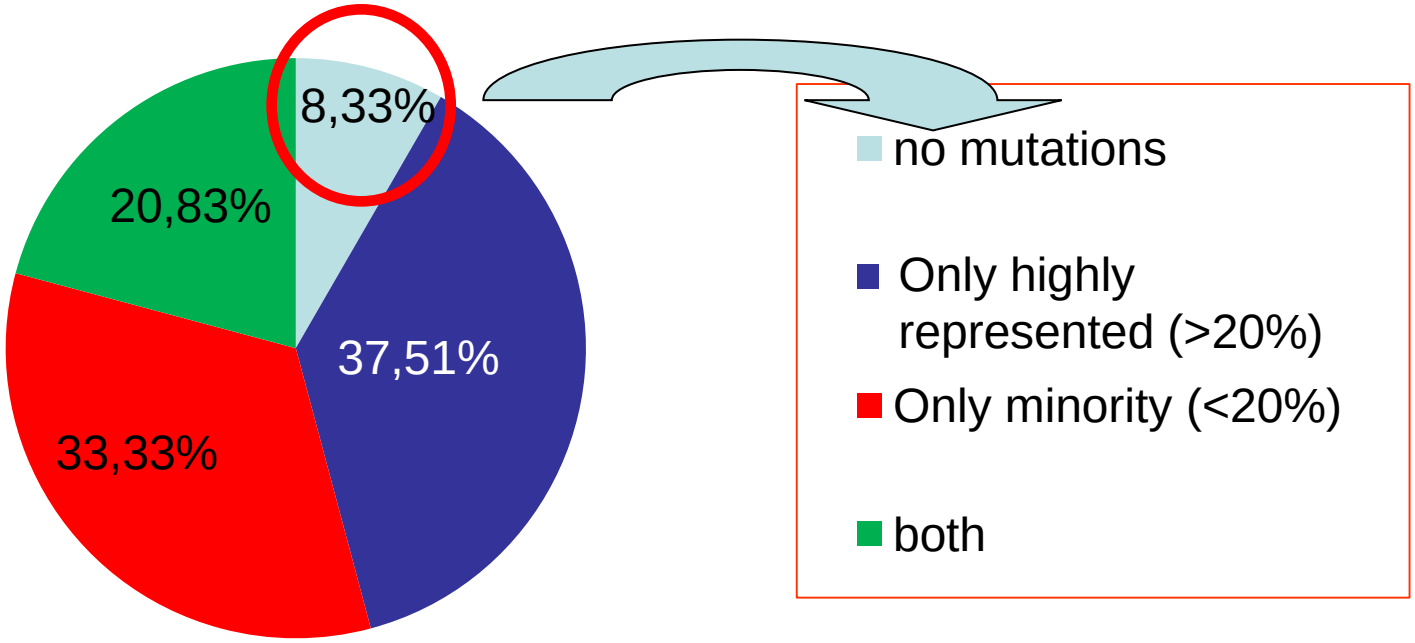
The vast majority of HCV naive patients harbour NS3 mutations



HCV resistance testing is still under scrutiny

- However, there is no agreement on the usefulness of performing resistance testing prior to initiate DAA-based therapy
- Even the use of resistance testing in failing cases is questioned, for a number of reasons
  - ✓not all virological failures are accounted for by the emergence of resistant variants
  - ✓the resistant variants rapidly decline (although not always disappear) after treatment stop
  - ✓the availability of different classes of drugs, and their combined use, may achieve an enormous rising of the genetic barrier to antiviral resistance, overcoming this problem

NGS may unravel viral dynamics in DAA failure and help to address this issue

NGS offers significant advantages for the application to clinical microbiology. At present, its use is mainly limited by

- high cost,
- bioinformatic analysis bottleneck,
- clinical validation

Work in progress

Paris, May 1888

I. Abbate
G. Rozera
B. Bartolini
} GS FLX experiments
} HIV studies

S. Menzo
M. Solmone
D.Vincenti
} HBV studies

E. Giombini
M. Selleri
} Bioinformatics

Clinical Division

G. D'Offizi
U. Visco-Comandini
} Management of patients

CINECA

A. Desideri
G. Chillemi
T. Biagini
} IT and software development