University of Sao Paulo

School of Pharmaceutical Sciences of Ribeirao Preto

**Development of a DNA microarray platform for the detection of
viruses transmitted by small mammals and arthropods**

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Ribeirao Preto

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University of Sao Paulo

School of Pharmaceutical Sciences of Ribeirao Preto

**Development of a DNA microarray platform for the detection of
viruses transmitted by small mammals and arthropods**

Doctorate thesis presented to the Post-graduate Program of Biosciences Applied to Pharmacy to obtain the title of Doctor of Sciences.

Field: Biosciences Applied to Pharmacy

Student: Mohd Jaseem Khan

Supervisor: Victor Hugo Aquino Quintana

Ribeirao Preto

2015

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TO MY PARENTS AND BROTHERS

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ABSTRACT

Khan, M. J. **Development of a DNA microarray platform for the detection of viruses transmitted by small mammals and arthropods.** 197p. Thesis (Doctoral). School of Pharmaceutical Sciences of Ribeirao Preto – University of Sao Paulo, 2015.

Human activities have been responsible for the global environmental changes, resulting in an increase number of incident of vector- and rodent-borne diseases worldwide. Rodents and arthropods-borne viruses are important globally emerging and re-emerging viruses and most of them are RNA viruses. Efficient and early diagnosis of these infections are very important to prevent their spread, to improve clinical management of the patients, as well to protect livestock and domestic animals. Currently, available diagnostic methods such as immunoassays, polymerase chain reaction and virus isolation can detect only one or few viruses in a single assay. The DNA microarray platform has emerged as diagnostic tool suitable for high throughput screening of pathogenic agents. The aim of this study was to develop a DNA microarray platform (RoboArboVirusChip) for detecting rodent- and arthropod-borne viruses, which belong to seven families: *Bunyaviridae* (genera *Orthobunyavirus*, *Nairovirus* and *Phlebovirus*), *Flaviviridae* (genus *Flavivirus*), *Togaviridae* (genus *Alphavirus*), *Reoviridae* (genera *Orbivirus*, *Seadornavirus* and *Coltivirus*), *Rhabdoviridae* (genera *Vesiculovirus* and *Ephemerovirus*), and *Asfarviridae* (genus *Asfarvirus*). Specific oligonucleotide probes of 60-mer (n=4209) targeting 412 virus species and generic probes of 25-35-mer (n=87) targeting viruses at the genus level were designed. A total of 17 reference viruses belonging to the *Bunyaviridae*, *Flaviviridae*, *Rhabdoviridae* and *Togaviridae* families were used to standardize RoboArboVirusChip. All reference viruses were specifically detected without any cross hybridization; however, the generic probes were not able to identify the viruses at the genus level. The RoboArboVirusChip was able to specifically identify four viruses contained in three different mixtures: i) virus of different families, ii) virus of the *Flavivirus* genus, and iii) the *Dengue virus* (DENV) serotypes. The four DENV serotypes were used to evaluate the sensitivity of the RoboArboVirusChip, which was able to detect a minimum of 25 RNA copies/mL of the viruses, confirming its high sensitivity. The applicability of the RoboArboVirusChip to detect viruses in clinical samples was tested with serum samples obtained from dengue suspected cases (four positive cases and 40 negative cases). DENV was detected in the four positive serum samples, while in the 40 negative serum samples, it was not detected any virus. The results obtained in this study suggest that the RoboArboVirusChip platform could be a useful tool for early diagnosis of robovirus and arbovirus infections during epidemic outbreaks, helping in the rapid implementation of disease containment strategies.

Keywords: Virus, small mammals, arthropods, DNA microarray.

RESUMO

Khan, M. J. **Desenvolvimento de uma plataforma de microarranjo de DNA para detecção de vírus transmitidos por pequenos mamíferos e artrópodes..** 197f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2015.

As atividades humanas têm sido responsável por mudanças ambientais globais, resultando num aumento do número de casos de doenças transmitidas por vetores e roedores em todo o mundo. Os vírus transmitidos por roedores e artrópodes são vírus emergentes e re-emergentes de importância global, sendo que a maioria deles são vírus de RNA. O diagnóstico eficiente e precoce dessas infecções são muito importantes para evitar a sua propagação, para melhorar o manejo clínico dos pacientes e também, para proteger o gado e os animais domésticos. Atualmente, os métodos de diagnóstico disponíveis, tais como os imunoensaio, a reação em cadeia da polimerase e o isolamento viral podem detectar apenas um ou poucos vírus em um único ensaio. A plataforma de microarranjo de DNA tem surgido como uma ferramenta de diagnóstico apropriada para o monitoramento em larga escala de agentes patogênicos. O objetivo deste estudo foi desenvolver uma plataforma de microarranjo de DNA (RoboArboVirusChip) para a detecção de vírus transmitidos por roedores e artrópodes, os quais pertencem a sete famílias: *Bunyaviridae* (gêneros *Orthobunyavirus*, *Nairovirus* e *Phlebovirus*), *Flaviviridae* (gênero *Flavivirus*), *Togaviridae* (gênero *Alphavirus*), *Reoviridae* (gênero *Orbivirus*, *Seadornavirus* e *Coltivirus*), *Rhabdoviridae* (gêneros *Vesiculovirus* e *Ephemerovirus*), e *Asfarviridae* (gênero *Asfarvirus*). Sondas oligonucleotídicas de 60-mer (n=4209) específicas contra 412 espécies virais, e sondas genéricas de 25-35-mer (n=87) para detecção de vírus a nível do gênero foram desenhados. Um total de 17 vírus de referência, pertencentes às famílias *Bunyaviridae*, *Flaviviridae*, *Rhabdoviridae* e *Togaviridae* foram utilizados para padronizar o RoboArboVirusChip. Todos os vírus de referência foram detectados especificamente sem apresentação de hibridação cruzada, porém as sondas genéricas não foram capazes de detectar os vírus a nível do gênero. O RoboArboVirusChip foi capaz de identificar especificamente quatro vírus contidos em diferentes misturas: i) vírus de diferentes famílias, ii) vírus pertencentes ao gênero *Flavivirus*, e iii) os sorotipos do vírus da dengue (DENV). Os quatro sorotipos do DENV foram utilizados para determinar a sensibilidade do RoboArboVirusChip, o qual foi capaz de detectar um mínimo de 25 cópias de RNA/mL. A aplicabilidade do RoboArboVirusChip para detectar vírus em amostras clínicas foi avaliada testando amostras de soro de pacientes com suspeita de dengue (quatro casos positivos e 40 casos negativos). Os resultados obtidos neste estudo sugerem que o RoboArboVirusChip poderá ser uma ferramenta útil para o diagnóstico precoce da infecção causada por robovírus e arbovírus, auxiliando na rápida implementação de estratégias de contenção das doenças causadas por esses vírus.

Palavras chaves: Vírus, pequenos mamíferos, artrópodes, microarranjo de DNA.

RESUMEN

Khan, M. J. **Desarrollo de una plataforma de microarreglo de DNA para detección de virus transmitidos por pequeños mamíferos y artrópodos**. 2015. 197h. Tesis (Doctorado). Facultad de Ciencias Farmacéuticas de Ribeirao Preto – Universidad de San Pablo, Ribeirao Preto, 2015.

Las actividades humanas han sido responsables por cambios ambientales globales, resultando en un aumento del número de casos de enfermedades transmitidas por vectores y roedores en todo el mundo. Los virus transmitidos por roedores e artrópodos son virus emergentes e re-emergentes de importancia mundial, siendo que la mayoría son virus de RNA. El diagnóstico eficiente y rápido de esas infecciones son muy importantes para evitar su propagación, para mejorar la atención clínica de los pacientes y también, para proteger el ganado y a los animales domésticos. Actualmente, los métodos de diagnóstico disponibles, tales como los inmunoensayos, la reacción en cadena de la polimerasa y el aislamiento viral pueden detectar solo un o pocos virus en un único examen. La plataforma de microarreglo de DNA ha surgido como una herramienta de diagnóstico apropiada para monitorizar en larga escala de agentes patogénicos. El objetivo de este estudio fue desenvolver una plataforma de microarreglo de DNA para la detección de virus transmitidos por roedores e artrópodos, los cuales pertenecen a siete familias: *Bunyaviridae* (géneros *Orthobunyavirus*, *Nairovirus* e *Phlebovirus*), *Flaviviridae* (género *Flavivirus*), *Togaviridae* (género *Alphavirus*), *Reoviridae* (género *Orbivirus*, *Seadornavirus* y *Coltivirus*), *Rhabdoviridae* (géneros *Vesiculovirus* y *Ephemerovirus*), y *Asfarviridae* (género *Asfarvirus*). Sondas oligonucleotídicas de 60-mer (n=4209) específicas contra 412 especies virales y sondas genéricas de 25-35-mer (n=87) para la detección de virus a nivel del género fueron diseñados. Un total de 17 virus de referencia, pertenecientes a las familias *Bunyaviridae*, *Flaviviridae*, *Rhabdoviridae* y *Togaviridae* fueron utilizados para estandarizar el RoboArboVirusChip. Todos los virus de referencia fueron específicamente detectados sin la presencia de hibridación cruzada, sin embargo, las sondas genéricas no fueron capaces de detectar los virus a nivel del género. El RoboArboVirusChip fue capaz de identificar específicamente cuatro virus contenidos en diferentes mezclas: i) virus de diferentes familias, ii) virus pertenecientes al género *Flavivirus*, y iii) los serotipos del virus del dengue (DENV). Los cuatro serotipos del DENV fueron utilizados para determinar la sensibilidad del RoboArboVirusChip, el cual fue capaz de detectar un mínimo de 25 copias de RNA/mL. La aplicabilidad del RoboArboVirusChip para detectar virus en muestras clínicas fue evaluada analizando muestras de suero de pacientes con sospecha de dengue (cuatro casos positivos y 40 casos negativos). El DENV fue detectado en las cuatro muestras positivas, mientras que ningún virus fue detectado en las muestras negativas. Los resultados obtenidos en este estudio sugieren que el RoboArboVirusChip podrá ser una herramienta útil para el diagnóstico rápido de la infección causada por robovirus e arbovirus, ayudando en la rápida implementación de estrategias para la contención de las enfermedades causadas por esos virus.

Palabras claves: Virus, pequeños mamíferos, artrópodos, microarreglo de DNA.

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List of Abbreviations of reference viruses

BSQV	<i>Bussuquara virus</i>
CHIKV	<i>Chikungunya virus</i>
DENV-1	<i>Dengue virus type 1</i>
DENV-2	<i>Dengue virus type 2</i>
DENV-3	<i>Dengue virus type 3</i>
DENV-4	<i>Dengue virus type 4</i>
GUAV	<i>Goarua virus</i>
IGUV	<i>Iguape virus</i>
ILHV	<i>Ilheus virus</i>
MAYV	<i>Mayaro virus</i>
OROV	<i>Oropouche virus</i>
PIRYV	<i>Piry virus</i>
RIOMV	<i>Rio Mamore virus</i>
SLEV	<i>St.Louis encephalitis virus</i>
WNV	<i>West Nile virus</i>
YFV	<i>Yellow fever virus</i>

List of Abbreviations other than viruses

%	Percentage
>	Greater than
°C	Degree centigrade
Mg	Micro gram
μl	Micro liter
μM	Micrometer
Ab	Antibody
AmB	Amphotericin B
BLAST	Basic local alignment search tool
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribo Nucleic Acid
CLC	CLC Main Workbench

CMC	Carboxymethyl cellulose
CO ₂	Carbon dioxide
CPE	Cytopathic effect
Cy3	Cyanine Dye 3
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
dsDNA	Double-strand DNA
dsRNA	Double-strand RNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
G/C	Guanine/ Cytosine
ICTV	International Committee on the taxonomy of viruses
ICTVdB	International Committee on the taxonomy of viruses database
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
KCl	Potassium chloride
L15 medium	Leibovitz's L-15 medium
LLOD	Lower Limit of Detection
MAbs	Monoclonal antibodies
Mg ₂ SO ₄	Magnesium sulfate
MgCl	Magnesium chloride
MIAF	Mouse immune ascetic fluid
Min	Minutes
mL	Milli liter
MLS	Master Species List
mM	Milli molar
MMLV	Moloney murine leukemia virus
MQ	MilliQ water
NCBI	National Center for Biotechnology Information
NH ₄ SO ₄	Ammonium sulfate
Nm	Nanometer
NS3	Non-structutral protien 3
ORF	Open reading frame
PBS	Phosphate-buffered saline

PFU	Plaque forming unit
pH	Potential of hydrogen
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S1	Segment-1
S6	Segment-6
S10	Segment-10
SDS	Sodium dodecyl sulfate
SIA	Sequence-independent amplification
SSC	Saline-sodium citrate
ssRNA	Single-strand RNA
Taq	Thermus aquaticus DNA polymerase enzyme
TE	Tris EDTA
TAE	Tris Acetate EDTA
TPB	Tryptose phosphate broth
Tris-HCL	Tris- hydrogen chloride
Tris-SO4	Tris-sulfate
U	Unit
UV	Ultra Violet
VP2	Viral Protein-2
VP5	Viral Protein-5
WGA	Whole-genome amplification

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1. INTRODUCTION

Roboviruses (Rodent-borne viruses) belong to two different families: *Arenaviridae* (*Mammarenavirus* and *Reptarenavirus* genera) and *Bunyaviridae* (genera *Hantavirus*) (Gegúndez and Lledó, 2005). Approximately, ten mammarenaviruses are known to cause disease in human through natural or laboratory acquired infection. The clinical symptoms of four viruses (Guanarito, Junin, Lassa and Machupo) are mimic in several ways. Reptarenaviruses are Snake-infecting new arenavirus genera causes' fatal disease called Boid inclusion body disease (BIBD). There is no evidence of these viruses causing disease in human (Hetzel *et al.*, 2014). About twenty *Hantaviruses* are responsible for in Asia and Europe "Hemorrhagic fever with Renal Syndrome" (HFRS) and in Americas *Hantavirus* Cardiopulmonary Syndrome (HCPS) (Zapata and Salvato, 2013; Kruger *et al.*, 2014). They infect humans by the inhalation of aerosols contain virus, through ingestion of contaminated stored food or due to consumption contaminated rodents meat (Ter Meulen *et al.*, 1996). Arboviruses (arthropod-borne viruses) are one of the most important emergence and re-emergence agents nearly worldwide. The arboviruses belong to seven families: *Bunyaviridae* (genus *Orthobunyavirus*, *Nairovirus* and *Phlebovirus*), *Flaviviridae* (genus *Flavivirus*) and *Togaviridae* (genus *Alphavirus*). In addition, a small group of arboviruses are included in the families *Reoviridae* (genus *Orbivirus*, *Seadornavirus* and *Coltivirus*), *Rhabdoviridae*: (genres *Vesiculovirus* and *Ephemerovirus*), *Orthomyxoviridae* (gender *Thogotovirus*) and *Asfarviridae* (gender *Asfarvirus*) they cause a wide range of infections in humans, domestic and wild animals (Jones *et al.*, 2008; Weaver and Reisen, 2010). There are currently more than 550 arboviruses have been identified, mostly belong to families above mentioned. More than 150 viruses are responsible for flu-like to fatal disease in humans and 50 viruses pathogenic animals (Gao *et al.*, 2010; Cleton *et al.*, 2012; Gan and Leo, 2014; Hubálek *et al.*, 2014). For an example, only dengue (DENV) virus infections are at present roughly to cause over 100,000,000 clinical cases every year specific a re-emergence throughout the tropical and subtropical countries. Approximately 17 million human's deaths every year reported throughout the world. The arboviral infections are rapidly spreading and this is major public health issue. There are not specific and effective treatments are available for the roboviruses and arboviruses with deal for those symptoms supportive therapy has been used with exception of

Yellow fever (YFV) and Japanese encephalitis (JEV) vaccines (Cleton *et al.*, 2012; Bhatt *et al.*, 2013; Dash *et al.*, 2013). Mainly, the medically important bugs: Mosquitoes, ticks, midges and sandflies, are known vectors responsible for the inoculation of arboviruses during blood feed, through contaminated mouthparts they enter in the body of host (humans and animals). The viruses first amplify within the tissues salivary gland of arthropods to produce high concentration of virus and are then passed on to host (Gubler, 2001). Other, non-vector transmission through laboratory associated, organ transplantation, mother to child and breast feeding (Descoux, 2013). These viruses are mostly causing zoonosis in the wild and are maintained in nature in complex cycles involving birds, rodents, marsupials, primates and sloths as reservoirs and wild mosquitoes, which suck the blood of these animals as vectors. Among the animal reservoir, mostly migratory birds, are infected, and are important carriers of arboviruses to distant sites (Meslin, 1997). The introduction of humans or animals viremic at these sites could lead to the emergence of urban cycles. Regarding robivirus, their emergence is associated with environmental changes caused by intensive agriculture causing increased food availability, an increase in the population of wild rodents, including attracting these animals to living next to man, who is mainly virally infected, inhalation of fecal and urinary components excreted by these infected animals and some important factors are responsible for the arboviruses will continue to emerge in new regions: landscape composition, deforestation, floods, increasing surface temperatures, urbanization, travel and commerce like animals and their products (Zeier *et al.*, 2005; Dash *et al.*, 2013).

1.1. Review and Literature

1.2. Background of Roboviruses:-

The Robovirus term refers to Rodent-borne viruses (viruses transmitted by rodents) belonging to two families:

1. Family *Arenaviridae* (two genera *Mammarenavirus* and *Reptarenavirus*)
2. Family *Bunyaviridae* (only genus *Hantavirus*)

Both families of viruses are transmitted from rodents (natural reservoir host) to human beings (Gegúndez and Lledó, 2005).

Note: The genus Hantavirus we discussed in arbovirus section.

1.2.1. Arenaviruses:

1.2.2. Taxonomy

The first arenavirus prototype LCMV was isolated in 1933 by doing serial passage of SLEV virus infected material from 42 years old dead human body, in monkey's brain. Arenaviruses are the group of viruses that represents the family *Arenaviridae* and was named after the sandy (Latin, *arenosus*) appearance of the ribosomes originally seen in thin sections of virions (Armstrong, 1934).

The family *Arenaviridae* consists of two proposed genera *Mammarenavirus* and *Reptarenavirus* (Radoshitzky *et al.*, 2015). The *Mammarenavirus* (referred to “mammals and arenaviruses”) for the genus for all currently known mammalian viruses, which is currently subdivided into two major groups, New World (*Tacaribe virus*) and Old World (*Lymphocytic choriomeningitis virus*) mainly on the basis of geographical distribution (Old World, Eastern Hemisphere and New World, Western Hemisphere), serological and molecular properties (Charrel *et al.*, 2008). Both groups of arenaviruses currently contains twenty five virus species recognized by the International Committee for Taxonomy of Viruses Master-Species list 2014 (MSL#29) released on June, 2014 (Salvato *et al.*, 2012) as well as many newly discovered viruses whose taxonomic status has not yet been confirmed by the ICTV.

The Old World arenaviruses contain a total of sixteen viruses; seven species and nine tentative virus species. Thirty viruses, eighteen species, one strain and eleven tentative belongs to The New World viruses that were further divided into four lineages designated clades A, B, C, and A/Rec. Five viruses belonged to clade A; *Allpahuayo mammarenavirus* (ALLV), *Flexal mammarenavirus* (FLEV), *Parana mammarenavirus* (PARV), *Pichinde mammarenavirus* (PICV) and *Pirital mammarenavirus* (PIRV). Clade B contained eight species *Amapari mammarenavirus* (AMAV), *Chapare mammarenavirus* (CHAPV), *Cupixi mammarenavirus* (CPXV), *Guanarito mammarenavirus* (GTOV), *Junin mammarenavirus* (JUNV), *Machupo mammarenavirus* (MACV), *Sabia mammarenavirus* (SABV) and *Tacaribe mammarenavirus* (TCRV). Clade C is composed of four viruses: two species and two unclassified *Latino mammarenavirus* (LATV) and Oliveros virus (OLVV) and unclassified; *Pampa mammarenavirus* (PAMV) and *Pinhal mammarenavirus*. The clade A/Rec contained

total thirteen viruses *Bear Canyon mammarenavirus* (BCNV), *Tamiami mammarenavirus* (TAMV), *Whitewater Arroyo mammarenavirus* (WWAV); unclassified *Big brushy tank mammarenavirus* (BBTV), *Black Mesa mammarenavirus* (BMV), *Catarina mammarenavirus* (CTNV), *North American mammarenavirus* (NAAV), *Ocozocoautla de Espinosa mammarenavirus* (OCEV), *Real de Catorce mammarenavirus* (RDCV), *Skinner Tank mammarenavirus* (SKTV), *Tonto Creek mammarenavirus* (TTCV), *Black Mesa mammarenavirus*, *Orogrande mammarenavirus* and *Arenavirus H0380005 mammarenavirus*. The *Rio Carcarana mammarenavirus* virus status is still unclassified (see Table 1).

Table 1. Summary of viruses belongs to Genera: *Mammarenaviruses*

Serogroup	Species	Strains	Tentative	Total	Mouse	Rat	Bat	N/D	HF	N/K	Others
LASV- LCMV	9	-	7	16	8	6	-	2	3	13	-
Taciribe	18	1	11	30	8	19	1	2	6	24	-
Ungrouped	-	-	1	1	-	??	??	??	??	??	-
Total	27	1	19	47	16	25	1	4	9	37	-

1.2.3. Reservoir host in nature:-

Each virus is associated with either one species or a few closely related rodents with the exception of Tacaribe virus, which natural reservoir is bat. The New World rodents (rats and mice) belong to family *Muridae*, subfamily *Sigmodontinae*, while the Old World rodents belong to family *Muridae*, subfamily *Murinae*. Taken together, these types of rodents are located across the greater proportion of the earth's landmass, including Europe, Asia, Africa, and the Americas. One notable exception is Tacaribe virus, found in Trinidad in the Caribbean region, which was isolated from bats and mosquitoes during a rabies surveillance in Trinidad from, 1956 – 1958 (Downs *et al.*, 1963). In 2012, several arenaviruses have been isolated from non-poisonous snakes: Boa constrictors (*Boa constrictor*), an annulated tree snake (*Corallus annulatus*) and python with inclusion body disease-associated arenaviruses (BIBDAV) (Stenglein *et al.*, 2012). More recently, the re-isolation of the arenavirus from pool of one hundred host-seeking lone star ticks (*Amblyomma americanus*) was collected in a Florida in 2012 (Sayler *et al.*, 2014).

1.2.4. Transmission to Rodents:

The rodents normally infected with arenaviruses; however, they are asymptomatic, with chronic viremia and viruria, thus spreading viruses to other non-infected rodents and other mammals. Infected reservoir host sheds virus into the environment via urine, feces and saliva. Some New World arenaviruses transmitted among adult rodents, via fighting and inflicting bites. In addition, Old World arenaviruses can pass from mother rodents to their offspring during pregnancy (Vitullo *et al.*, 1987; Vitullo and Merani, 1988). New rodents in each host species infected with virus once, in many cases only in limited part of the geographical localization.

1.2.5. Transmission to Humans:

Arenaviruses infect humans by inhalation of aerosolized particles, through ingestion of contaminated stored food or consumption of their contaminated meat. (Ter Meulen *et al.*, 1996). However, there are some risk factors involved in the virus spread; for example, human infection is associated with agricultural work, as the infected rodents prefers a field or agricultural habitat. In areas where the rodent species habitat includes human homes or other buildings, infection occurs in domestic settings. This may occur in a variety of ways. Person-to-person transmission is associated with direct contact with the blood, saliva, semen, stool, tissues, urine, vomits, and other secretions or via inhalation of aerosolized virus from clinical specimens. Patient infected with Lassa virus may shed virus in urine and semen for several weeks after recovery, and it is documented that sexual activity has resulted in secondary transmission (McCormick *et al.*, 1987). Mother to child transmission is well evidenced and may result in severe congenital malformation or death of the fetus or newborn (Cj, 2000;). Transmission of lymphocytic choriomeningitis virus by organ transplantation was recently described (Ja, 2005;). Nosocomial transmission also reported. Contact with objects contaminated with these materials, such as medical equipment, nursed in open wards where patient is not properly isolated also associated with transmission (Leparc-Goffart and Emonet, 2011; Atkinson *et al.*, 2014).

Table 2. Summary of Arenaviruses pathogenic for human.

Virus	Type of disease	Annually incidence	Fatality rate	Reference
LCMV	Lymphocytic choriomeningitis	>5%	<1%	(Cj, 2000;)
Lassa virus	Lassa fever	≈2000000	15%	(Yun and Walker, 2012)
Junin virus	AHF	300 to 1000	15%–30%	(Enria <i>et al.</i> , 2008)
Machupo virus	Bolivian hemorrhagic fever	>200	20%	(Franz <i>et al.</i> , 1997)
Guanarito virus	Venezuelan hemorrhagic fever	≈618	23%	(Fulhorst <i>et al.</i> , 2008)
Sabia virus	Not yet named	1	fatal	(Lisieux <i>et al.</i> , 1994)
Whitewater Arroyo virus	Not yet named	3/3	fatal	(Charrel and De Lamballerie, 2010)
Lujo virus	Lujo hemorrhagic fever	4/5	80%	(Briese <i>et al.</i> , 2009)
Chapare virus	Not yet named	Unknown	Unknown	(Liu, 1974)

1.2.6. Recent epidemiological outbreak

In May 2015, fatal case of Lassa virus was reported the patient returning from Liberia to USA ([Http://Www.Cdc.Gov/Vhf/Lassa](http://www.Cdc.Gov/Vhf/Lassa), 2015).

1.2.7. Vaccines:

To date, there are no FDA approved vaccines are available to fight against arenavirus infections except Candid#1 for Junin virus but licensed for use only in Argentina (Cheng *et al.*, 2015; Rathbun *et al.*, 2015).

1.2.8. Antiviral drugs:

Currently, only licensed partially effective drug nucleoside inhibitor ribavirin is available for treatment of arenavirus infection associated with side effects (Cheng *et al.*, 2015).

1.2.9. Taxonomy of genus *Reptarenavirus*

In the 1970's, inclusion body disease first detected in the United States, where it was affecting many species of snakes in zoo and private museum. Based on cross-react by western blot with anti-Junín virus antibodies, comparison weak cross reactivity was

noted with anti-Machupo virus and anti-LCMV antibodies. The snake viruses do not able to react with antisera from patients with Bolivian hemorrhagic fever in ELISA. These new snake viruses may be hold the Old World and New World complexes of this family (Mj., 2012; Stenglein *et al.*, 2012; Hetzel *et al.*, 2013). A total seven viruses identified in the genus, three confirm species and four of them are tentative (Table 3).

Table 3. Summary of genus *Reptarenavirus*

Serogoup	Species	Strains	Tentative	Total	Mouse	Rat	Bat	N/K	HF	N/K
Alethinophid 1 Reptarenavirus	1	-	-	1	-	-	-	1	-	4
Alethinophid 2 Reptarenavirus	1	-	-	1	-	-	-	1	-	-
Alethinophid 3 Reptarenavirus	1	-	-	1	-	-	-	1	-	-
Ungrouped	-	-	4	4	-	-	-	4	-	-
Total	3	-	4	7		-	-	7	-	

1.2.10. Reservoir host in nature:

Not yet been confirmed (Hepojoki *et al.*, 2015).

1.2.11. Transmission:

Snakes consume of rodents and bats, and both can be infected by arenaviruses and filoviruses (Zapata and Salvato, 2013).

1.2.12. Disease in Human:

To date, no disease was reported in human (Hepojoki, J.2015).

Disease in Animals:

Fatal disease called Boid inclusion body disease (BIDB) in boid Snakes (Hepojoki, J.2015)

Epidemiology:

The geographical area is not yet cleared (Hepojoki, J.2015).

Treatment:

Not treatment available

Vaccine:

No vaccine.

1.2.13. Arbovirus:

The origin of “Arbovirus” for first time was using in laboratory jargon at in early 1940s from the joke like “you can’t see it, smell it, touch it, or culture it, but it can kill you”. The Cuban Dr. Carlos Finlay, first time proposed mosquito-vector theory in 1881 and the US Army Yellow fever commission head Major Walter Reed discovered first arbovirus, Yellow fever virus in 1901 (Russell, 1934; Reeves, 2001; Chaves-Carballo, 2005). In 1930, six viruses (African swine fever, Blue tongue, Louping ill, Nairobi sheep disease, Vesicular stomatitis and Yellow fever) were known caused disease in human and transmitted by arthropods and today more than 550 known.

Arboviruses (an acronym for “arthropod-borne viruses”) are one of the most important emergence and re-emergence agents nearly worldwide. They cause a wide range of infections in humans and domestic animals (Jones *et al.*, 2008). They are able to replicate and transmitted by medically important bugs such as mosquitoes, ticks, midges and sand flies, are known vectors responsible for the inoculation of arboviruses during blood feed (Gubler, 2001). These arboviruses must replicate biologically in the arthropod vector prior to transmission (Weaver, 1997). As, mechanically transmitted, without replication in the vector, Biological transmission can be vertical, involving the passage of the virus from an infected female vector to both male and female offspring. Horizontal transmission can be venereal, from a vertically infected male directly to a female vector, as well as oral from a female vector to a vertebrate host via the saliva during blood feeding. The latter horizontal mode of transmission is most common for the majority of arboviruses and involves infection of the vector alimentary tract following a viremic blood meal, dissemination of the virus in the vector, and virus replication in the salivary glands, followed by the injection of infectious saliva during blood feeding (Weaver and Reisen, 2010).

1.2.14. Taxonomy:

The arboviruses belong to seven taxonomic families: *Bunyaviridae* (genera *Orthobunyavirus*, *Nairovirus* and *Phlebovirus*), *Flaviviridae* (genus *Flavivirus*) and *Togaviridae* (genus *Alphavirus*). In addition, a small group of arboviruses are included in the families *Reoviridae* (genera *Orbivirus*, *Seadornavirus* and *Coltivirus*), *Rhabdoviridae*: (genera *Vesiculovirus* and *Ephemerovirus*), *Orthomyxoviridae* (genus *Thogotovirus*) and *Asfarviridae* (genus *Asfarvirus*) they cause a wide range of infections in humans, domestic and wild animals (Jones *et al.*, 2008; Weaver and Reisen, 2010).

Table 4. Taxonomic status of known and tentative arboviruses

Family	Genera within family	Genera belongs to Arboviruses	Number of viruses	Number of viruses pathogenic for Human	Number of viruses pathogenic for Livestock's
<i>Bunyaviridae</i>	5	3	286	49	12
<i>Flaviviridae</i>	4	1	76	38	13
<i>Togaviridae</i>	2	1	36	16	9
<i>Reoviridae</i>	15	3	171	8	8
<i>Rhabdoviridae</i>	11	2	37	7	6
<i>Orthomyxoviridae</i>	6	1	6	0	1
<i>Asfarviridae</i>	1	1	1	0	1
Total	44	12	613	118	50

1.2.15. History and classification of the family *Bunyaviridae*

The family *Bunyaviridae* name derived from *Bunyamwera virus* (BUNV). This was isolated during an investigation of Yellow Fever in 1943 from *Aedes* spp. mosquitoes in the Semliki forest, Uganda. The family *Bunyaviridae* was officially recognized in 1975, which is the biggest and most diverse RNA viruses family (Elliott, 2014). According to the International Committee on Taxonomy of Virology (ICTV) currently this family is subdivided into five genera: *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Hantavirus* and *Tospovirus*. First three genera are pathogenic for humans and animals, fourth only for humans, *Hantaviruses*, which are mainly carried by small mammals. Genus *Tospovirus* are also transmitted by plant-feeding arthropods (thrips), but they infect plant, such as tomato and watermelon, in this case non-vertebrates (Briese *et al.*, 2013). Approximately, 469 arboviruses are known, of which 381 strains/unclassified mainly representing from four genera are *Orthobunyaviruses*, *Phleboviruses*, *Nairoviruses* and *Hantavirus*.

1.2.16. Genus *Orthobunyavirus*: History and taxonomy

To avoid confusion during discussion of any virus belonging to the family *Bunyaviridae*, the original *Bunyavirus* genus name it was changed to *Orthobunyavirus* in 1995 (Regenmortel, 2000). This genus is the largest genus within the family *Bunyaviridae*, with more than 170 viruses. Currently 48 viruses are species recognized by the ICTV, includes 122 viruses strain/ isolates, which is distributed throughout the world (Elliott, 2014). Most of the viruses are distributed into 18 different serogroups or left unclassified (Table 5). However, the historical term “serogroup” is not more useful for the viral taxonomy (Ladner *et al.*, 2014).

Table 5. Summary of arthropods-borne viruses of genus *Orthobunyavirus*

Serogroup	Species	Strains	Total	Tentative	Mosq	Sf	Ticks	M+S	ND	Flu-like	Enc	HF	N/K
Anopheles A	2	7	9	-	9	-	-	-	-	-	-	-	9
Anopheles B	1	1	2	-	2	-	-	-	-	-	-	-	2
Bakau	1	4	5	-	3	-	-	-	2	-	-	-	5
Bunyamwera	5	24	29	-	27	-	-	2	-	8	1	1	19
Bwamba	1	1	2	-	2	-	-	-	-	1	-	-	1
California	1	15	16	-	16	-	-	-	-	3	4	-	9
Capim	5	3	8	-	8	-	-	-	-	-	-	-	8
Gamboa	2	2	4	-	4	-	-	-	-	-	-	-	4
Group C	4	9	13	-	13	-	-	-	-	7	-	-	6
Guama	5	7	12		11	-	-	-	1	2	-	-	10
Koongol	1	1	2	-	2	-	-	-	-	-	-	-	2
Minatitlan	1	1	2	-	1	-	-	-	1	-	-	-	2
Nyando	1	1	2	-	2	-	-	-	-	-	-	-	2
Olifantsvlei	2	3	5	-	5	-	-	-	-	-	-	-	5
Patois	3	4	7	-	5	-	1	-	1	-	-	-	7
Simbu	12	22	34	-	16	6	-	5	7	1	-	-	33
Tete	0	4	4	-	-	1	2	-	1	-	-		4
Turlock	0	1	1	-	1	-	-	-	-	-	-	-	1
Wyeomyia	1	7	8	-	8	-	-	-	-	1	1	-	6
Unclassified	0	5	5	-	2	-	1	-	2	-	-	-	5
Total	48	122	170		137	7	4	7	15	23	6	1	140

1.2.17. Arthropod vectors:

The mosquitoes are main vector for this genus; mosquitoes transmit one hundred thirty seven viruses (137), sandflies (commonly referred to as "culicoid midges") seven (7), mosquitoes and sandflies both are responsible for seven (7) virus, through ticks four (4) and for fifteen (15) viruses vectors are still unknown (Table 5 and supplementary Table 3).

1.2.18. Vertebrate hosts:

Rodents, ruminants, sloths primates and birds as reservoirs (Jfst, 1998; Blitvich *et al.*, 2012).

1.2.19. Human disease:

At least 30 orthobunyaviruses are responsible for flu-like to hemorrhagic fever disease and 140 viruses non-pathogenic for humans. A total 23 viruses, eight from Bunyamwera group, seven from group C, three from California group, two from Guama group, one from each Bwamba, Simbu and Wyeomyia groups are responsible for flu-like illness. Encephalitis viruses belong mainly to three groups: California 4, Bunyamwera 1 and one from Wyeomyia. *Garissa virus* (Ngari complex) that belongs to Bunyamwera group causes hemorrhagic fever.

1.2.20. Animal disease:

There are eight orthobunyaviruses responsible for disease in animals. Two viruses from California group; *La Cross virus* caused encephalitis in dogs (Black *et al.*, 1994) and Snowshoe hare virus known for equine encephalitis (Heath *et al.*, 1989). The same number from Bunyamwera group; *Cache Valley virus* agent of an embryonic and fetal death of sheep, possibility of for all ruminants (Edwards, 1994) and *Main Drain virus* as a cause of equine encephalomyelitis (Emmons *et al.*, 1983). Four viruses from Simbu group affects livestock's ; *Akabane virus* is an exotic agent for severe congenital defects (Kirkland, 2002), *Aino virus* is associated with fetal defects in sheep and cattle (Noda *et al.*, 1998), *Schmallenberg virus* manifesting the CNS of sheep and cattle (Wu *et al.*, 2014) and *Shuni virus* causes of neurological disorders in horses (Van Eeden *et al.*, 2014).

1.2.21. Epidemiology

Viruses of the *Orthobunyaviruses* are reported worldwide (Lowen and Elliott, 2005).

1.2.22. Treatment:

No specific therapies for the *Orthobunyaviruses* (Hollidge *et al.*, 2012).

1.2.23. Vaccine:

No licensed vaccines are available for *orthobunyaviruses* those extremely effecting humans (Hollidge *et al.*, 2012).

1.2.24. Genus *Phleboviruses*: History and taxonomy

The genera *Phlebovirus* name came from *Phlebotomine* sandflies because most of virus members are transmitted by phlebotomine except Uukuniemi and Bhanja serogroups (Tesh, 1988). The genera *Phlebovirus* contains more than 80 viruses, with 28 confirmed classified into 9 antigenic complexes groups and 32 as tentative and unclassified complexes. Before 1991, Uukuniemi-like group was in a separate genus. (Table 6).

Table 6. Summary of genus *Phloboviruses*

Serogroups	Species	Strains	Tentative	Total	Sandflies	Mosq	SF+M	Ticks	ND	Flu-like	N/K	Others
Bujaru	-	-	5	5	5	-	-	-	-	1	4	-
Candiru	1	-	6	7	-	-	-	7	-	4	2	SFTS
Chilibre	1	5	7	13	4	1	-	-	8	1	12	-
Frijoles	1	1	-	2	2	-	-	-	-	-	2	-
Punta Toro	-	2	-	2	-	-	1	-	1	-	2	-
Rift Valley	1	2	1	4	3	1	-	-	-	2	2	-
Salehabad	4	5	21	30	17	3	1	-	9	4	24	RVF,M
SFNV	1	-	-	1	1	-	-	-	-	1	-	-
Uukuniemi	1	13	-	14	-	-	-	11	3	-	14	-
Unclassified	-	-	2	2	-	-	-	-	2	-	2	-
Total	10	28	42	80	32	5	2	18	23	13	64	3

1.2.25. Arthropod vectors:

Genus can divide into two groups Sandfly fever (transmitted by Sandfly and mosquitoes) and Uukuniemi like virus (transmitted by ticks), as we know, the genus name came from *Phlebotomine* sandflies. Thirty-two viruses transmitted by sandflies, 18 viruses through ticks, and five by mosquitoes. Mosquitoes and sandflies are responsible for two viruses and 23 viruses by unknown vectors (Table 6).

1.2.26. Vertebrate hosts:

The ruminants, rodents and bat are main reservoir host in nature (Olive *et al.*, 2012).

1.2.27. Human disease:

The fatal best-known virus from phlebovirus is Rift Valley fever virus and other three important pathogens include sandfly fever Naples, sandfly Sicilian and Toscana virus. Thirteen viruses are causes flu-like problem and 64 viruses still safe for human health.

1.2.28. Animal disease:

Rift Valley fever causes severe disease with more than 70% mortality rate in young animals like lamb and calf. The goat and buffaloes are also face problem with 10% mortality. Other Bhanja virus also pathogenic for the same animals and causes fever and meningoencephalitis.

1.2.29. Epidemiology:

Presente worldwide (Lantez *et al.*, 2011).

1.2.30. Treatment:

No therapies against the phlebovirus disease (Sidwell, R. W 1992).

1.2.31. Vaccine:

No vaccine (Sidwell, R. W 1992).

1.2.32. Genus *Nairovirus*: History and Taxonomy

The genus *Nairovirus* is named for Nairobi sheep disease (NSD), which was originally isolated near Nairobi, Kenya in 1910 as acute hemorrhagic gastroenteritis cases of sheep (Davies *et al.*, 1978). There are thirty-six viruses, seven species confirmed and 29 strains. The genus *Nairovirus* is divided into seven serogroups (Table 7).

Table 7. Summary of genus *Nairovirus*.

Serogroups	Species	Strains	Total	Ticks	T+SF	T+SF+M	Mosq	ND	Flu-like	N/K	Others
CCHF	1	2	3	2	1	-	-	-	-	2	CCF
Dera Ghazi Khan	1	5	6	5	-	-	-	1	-	6	-
Hughes	1	9	10	10	-	-	-	-	-	10	-
Nairobi Sheep Dis	1	3	4	4	-	-	-	-	2	2	-
Qalyub	1	3	4	4	-	-	-	-	-	4	-
Sakhalin	1	6	7	7	-	-	-	-	-	7	-
Thaifora	1	1	2	-	-	-	-	2	-	2	-
Total	7	29	36	32	1	0	0	3	2	33	1

1.2.33. Arthropod vectors:

The ticks are main vector for *Nairoviruses*; thirty-two viruses are use ticks as main vector, one virus is involved with ticks and sandflies and the vector of three viruses still not confirmed.

1.2.34. Vertebrate hosts:

The goat, sheep, seabirds and rats are vertebrate hosts (Hubálek *et al.*, 2014).

1.2.35. Human disease

Only three viruses from this genus have been identified as human pathogens, two viruses belong to *Nairobi sheep disease virus* serogroup causes flu-like symptoms and third *Crimean-Congo hemorrhagic fever virus* severe fatal with up to 50% mortality (Flusin *et al.*, 2011).

1.2.36. Animal disease

Gunjan virus also known as *Nairobi sheep disease virus* an important disease of ruminants and other *Soldado virus* can affect young seabirds (Hubálek *et al.*, 2014).

1.2.37. Epidemiology

Worldwide (Hubálek *et al.*, 2014).

1.2.38. Treatment

No effective treatment against these (Hubálek *et al.*, 2014).

1.2.39. Vaccine

No vaccines are available for Nairoviruses (Hubálek *et al.*, 2014).

1.2.40. History and taxonomy of genus *Hantavirus*:

The *Hantavirus* genus was named after the Hantaan river where *Hantaan virus* was discovered, previously known as Korean hemorrhagic fever, epidemic hemorrhagic fever was first come to light during 1951 to 1953, when approximately 3,200 cases reported among United Nations forces in Korea (Schmaljohn and Hjelle, 1997). This genus divided into four serotypes with total 97 viruses 38 viruses from OW and 59 representative from NW. A twenty-four confirmed species, seven strains and sixty-six still are tentative (Table 8).

Table 8. Summary of genus *Hantavirus*.

Serogroups	Species	Strains	Tentative	Total	OW	NW	HFRS	HCPS	Flu-like	N/K	Others
Hantaan	12	2	1	15	11	4	7	2	-	6	-
Puumala	1	1	-	2	2	-	-	-	1	1	-
Sin Nombre	8	4	11	23	-	23	-	15	1	6	HARDS
Thottapalayam	1	-	-	1	1	-	-	-	-	1	-
Ungrouped	2	-	54	56	24	32	3	6	-	47	-
Total	24	7	66	97	38	59	10	23	2	61	1

1.2.41. Mode of transmission

Mode of transmission is same like arenaviruses. Many Hantaviruses affect persons of low socioeconomic condition due to of poor housing conditions (Schmaljohn and Hjelle, 1997).

1.2.42. Rodent's vectors:

Hantaviruses are transmitted by various species of rodents (Khan and Khan, 2003).

1.2.43. Vertebrate hosts:

Small mammals specially, rodents are vertebrate hosts of Hantavirus (Jonsson *et al.*, 2010).

1.2.44. Human disease

The Hantaviruses are causes two types of disease in humans, 10 viruses are known to haemorrhagic fever with renal syndrome (HFRS) in old world and 23 viruses are responsible for hantavirus pulmonary syndrome (HPS) in new world. Two viruses are show flu-like problem, one from Sin Nombre group causes Hantavirus Associated Respiratory Distress Syndrome (HARDS) and 61 viruses are non-pathogenic or unknown.

1.2.45. Animal disease

Not reported.

1.2.46. Epidemiology:

Hantaviruses are presents in Europe and Asia, and Americas (Escutenaire and Pastoret, 2000).

1.2.47. Treatment:

No specific treatment available (Bellomo *et al.*, 2015).

1.2.48. Vaccine:

No licensed vaccines are available (Beltrán-Ortiz, C. E.2015).

1.2.49. Family *Flaviviridae*; History and classification

In 1881s, The Cuban Dr. Carlos Finlay first time suggested that mosquitoes were responsible to propagation of Yellow fever virus (an alternative name “vomito negro”) in vertebrates host. The Walter Reed Yellow Fever Commission proved this hypothesis around 1900 (Chiong, 1989). This family currently includes four different genera.

Table 9. Genera of the family *Flaviviridae*.

Genus	Meaning in Latin/Greek	Mode of Transmission	Reference
Flavivirus	Latin flavus “Yellow”	Arthropods	(Kuhn <i>et al.</i> , 2002)
Pestivirus	Latin pestis “Plague”	Direct contact	(Lanyon and Reichel, 2014)
Hepacivirus	Greek hepar, hepatos “Liver”	Blood-borne	(Romero-Brey and Bartenschlager, 2014)
Pegivirus	Persistent GB virus	Blood-borne	Stapleton, J. T. 2011

The *Flavivirus* genus is the largest, composed of 76 species divided into 12 groups. Of these groups, the Japanese encephalitis group contains the largest number of viral species associated with human disease. The majority of human infections following exposure to a mosquito vector in this group of viruses include the *Japanese encephalitis virus* (JEV), *Murray Valley encephalitis virus* (MVEV), *St. Louis encephalitis virus* (SLEV), *Dengue virus* (DENV) and *West Nile virus* (WNV). Other important ticks-borne group causes encephalitis called mammalian tick-borne (Table 10).

Table 10. Summary of genus *Flavivirus*.

Serogroup	Species	Strains	Tentative	Total	Ticks	Mosq	T+M	ND	Flu-like	Ence	HF	N/K
Aroa virus	1	-	-	1	-	1	-	-	-	-	-	1
Dengue virus	5	-	-	5	-	5	-	-	4	-	-	1
Entebbe bat	4	-	-	4	-	-	-	4	-	2	-	2
Japanese encephalitis	8	-	-	8	-	8	-	-	1	4	-	2
Kokobera	1	-	1	2	-	2	-	-	1	-	-	2
Mammalian tickborne	8	8	3	19	18	-	1	-	-	14	1	4
Modoc virus	3	-	-	3	-	-	-	3	-	-	-	3
Ntaya	7	1	-	8	-	7	-	1	1	1	-	6
Rio Bravo	6	-	1	7	-	-	-	7	2	-	-	5
Seabird tickborne	4	-	-	4	3	-	1	-	1	-	-	3
Spondweni	1	1	-	2	-	2	-	-	2	-	-	-
Yellow fever	9	1	-	10	-	8	-	2	3	-	1	6
Ungrouped	-	-	3	3	-	2	-	1	-	-	-	3
Total	57	11	8	76	21	35	2	18	15	21	2	38

1.2.50. Arthropod vectors:

Responsible vectors for genus *Flaviviruses* transmitted to vertebrates are variety of mosquitoes and ticks. The major role play by infected mosquitoes with 35 viruses, ticks are known to transmit 21 viruses and two viruses are transmitted by both vectors. Also, non-vector-borne four flaviviruses have been isolated from bats and rodents.

1.2.51. Vertebrate hosts

The vertebrate amplifier hosts of *flaviviruses* are Birds, sheep, pigs, rodents, camels and primates (Rosen, 1986; Mlera *et al.*, 2014).

1.2.52. Human disease:

Twenty-one *flaviviruses* are responsible for encephalitis and two viruses for hemorrhagic fever and 15 viruses are causes flu-like symptoms in humans (Table 10).

1.2.53. Animal disease:

Thirteen viruses have been reported to pathogenic for the animals, following viruses are infects animal; *Yellow fever virus*; monkeys, *Japanese encephalitis virus*; sows, boars and horses, *Murray Valley encephalitis virus*; horse and sheep, *West Nile virus*; equids and avian groups, *Usutu virus*; birds, *Israel turkey meningoencephalitis virus*; adult turkeys, *Tembusu virus*; ducks, *Wesselsbron virus*; sheep, goat, pig and horses, *Louping ill virus*; sheep, cattle, pig, dog and horses, *Tick-borne encephalitis virus*; birds, *Omsk hemorrhagic fever virus*; birds, *Kyasanur Forest disease virus* and *Tyuleniy virus* infect monkeys (Hubálek *et al.*, 2014).

1.2.54. Epidemiology:

Worldwide except Antarctica (Huang *et al.*, 2014).

1.2.55. Treatment:

No antiviral drugs are approved for use against the *flaviviruses* (Leyssen *et al.*, 2008).

1.2.56. Vaccine

Licensed vaccines are available for YEV and JEV to protect human and WNV lineage 1 are for horses in the United States and in Europe (Hills *et al.*, 2014; Beck and Barrett, 2015).

1.2.57. Family *Reoviridae*; History and classification

Sabin first introduced family *Reoviridae* name derived from **R**espiratory **E**nteric **O**rphan viruses in 1959 and originally these viruses are classified as echo virus 10 (Urbano and Urbano, 1994). Currently, the family *Reoviridae* contains fifteen genera divided into two subfamilies *Spinareovirinae* and *Sedoreovirinae* bases rectified International Committee for Taxonomy of Virology (ICTV) in 2009. Viruses belong to three genera; *Coltivirus*, *Orbivirus* and *Seadornavirus* transmitted by arthropods and infect animals.

Table 11. Summary of family *Reoviridae*.

Genus	Mode of transmission	Infect	Reference
Aquareovirus	Water	Fish and Mollusks	(Mohd Jaafar <i>et al.</i> , 2008)
Cardoreovirus	Water	Crustaceans	(Fan <i>et al.</i> , 2013)
Coltivirus	Arthropods	Mammals	(Klasco, 2002)
Cypovirus	Insect	Insects	(Li <i>et al.</i> , 2006)
Dinovernavirus	Arthropods	Insects	(Auguste <i>et al.</i> , 2015)
Fijivirus	Insect	Plant and Insects	(Soo <i>et al.</i> , 1998)
Idnoreovirus	Insect	Insects	(Miyazaki <i>et al.</i> , 2008)
Mimoreovirus	Water	Algae	(Attoui <i>et al.</i> , 2006)
Mycoreovirus	??	Fungi	(Eusebio-Cope and Suzuki, 2015)
Orbivirus	Arthropods	Mammals	(Drolet <i>et al.</i> , 2015)
Orthoreovirus	Fecal-oral route	Mammals, birds and reptiles	(Thimmasandra Narayanappa <i>et al.</i> , 2015)
Oryzavirus	Arthropods	Plants and Insects	(Auguste <i>et al.</i> , 2015)
Phytoreovirus	Insect	Plants and Insects	(Ji <i>et al.</i> , 2011)
Rotavirus	Fecal-oral route	Mammals and birds	(Dennehy, 2015)
Seadornavirus	Arthropods	Humans and Insects	(Attoui <i>et al.</i> , 2005)

Viruses belong to three genera; *Coltivirus*, *Orbivirus* and *Seadornavirus* transmitted by arthropods and infect animals are consider arboviruses.

1.2.58. History and taxonomy of genus coltivirus:

The genus *Coltivirus* name has been derived from *Colorado tick fever virus* (Urbano and Urbano, 1994). Currently, genus contains two species (Table 12).

Table 12. Summary of genus *Coltivirus*.

Serogroup	Species	Strain	Tentative	Total	Ticks	Flu-like	N/K
Colorado tick fever virus	2	-	-	2	2	1	1
Total	2	0	0	2	2	1	1

1.2.59. Arthropod vectors:

Ticks are main vector for *coltiviruses*, Rocky Mountain wood tick is transmitted *Colorado tick fever virus* (Table 12)

1.2.60. Vertebrate hosts:

Viruses maintains in many animals like chipmunks, deer, squirrels, wild mice, wood rats, wild rabbits , rodents and sheep (Attoui *et al.*, 2005).

1.2.61. Human disease:

Colorado ticks fever virus causes an acute systemic fever illness and Eaych virus

1.2.62. Animal disease:

Not reported yet.

1.2.63. Epidemiology

Colorado tick fever virus reported in USA and *Eaych virus* in Europe and Asia (Attoui *et al.*, 1998; Charrel *et al.*, 2004).

1.2.64. Treatment:

No antiviral treatment is available against coltiviruses except supportive care (Romero and Simonsen, 2008).

1.2.65. Vaccine:

No licensed vaccine available (Romero and Simonsen, 2008).

1.2.66. Genus Orbivirus; History and taxonomy

The genus currently, includes 166 viruses; 22 species (representing as serogroups), 132 strains and 12 unclassified (Table 13).

Table 13. Summary of genus *Orbivirus*

Serogroup	Species	Strains	Tentative	Total	Ticks	Mosq	Sandflies	N D	Flu- like	Ence	N/K
AHSV	1	8	-	9	-	-	9	-	-	-	9
BTV	1	25	-	26	-	-	26	-	-	-	26
Changuinola	1	11	-	12	2	1	6	3	1	-	11
Chenuda	1	6	-	7	7	-	-	-	-	-	7
Chobar Gorge	1	1	-	2	2	-	-	-	-	-	2
Corriaparta	1	5	-	6	-	6	-	-	-	-	6
EHD	1	8	-	9	-	-	9	-	-	-	9
EEV	1	6	-	7	-	-	7	-	-	-	7
Eubenangee	1	2	-	3	-	3	-	-	-	-	3
Great Island	1	35	-	36	36	-	-	-	1	-	35
Ieri	1	2	-	3	-	3	-	-	-	-	3
Kemerovo	1	1	-	2	2	-	-	-	-	-	2
Lebombo	1	-	-	1	-	1	-	-	-	-	1
Orungo	1	3	-	4	-	2	-	2	4	-	-
Palyam	1	12	-	13	3	2	5	3	-	-	13
PHSV	1	-	-	1	-	1	-	-	-	-	1
SCRV	1	-	-	1	1	-	-	-	-	-	1
Umatilla	1	3	-	4	-	4	-	-	-	-	4
WALV	1	2	-	3	-	-	3	-	-	-	3
Warrego	1	2	-	3	-	-	3	-	-	-	3
Wongorr	1	-	-	1	-	1	-	-	-	-	-
YUOV	1	-	-	1	-	1	-	-	-	-	1
Unclassified	-	-	12	12	2	8	-	2	-	-	12
Total	22	132	12	166	55	33	68	10	6	-	160

1.2.67. Arthropod vectors:

Transmitted by range of hematophagous arthropods; mainly, 68 viruses by sandflies, 55 through ticks, mosquitoes responsible for 33 and 10 viruses with unknown vectors.

1.2.68. Vertebrate hosts:

The genus orbivirus has a wide vertebrate host range includes domestic and wild ruminants, equines, marsupials, sloths, bats, birds (Belaganahalli *et al.*, 2015).

1.2.69. Human disease:

This genus not life threatening for human, only six viruses associated with human health and causes flu-like problem and 160 viruses non-pathogenic (Table 13).

1.2.70. Animal disease:

Eight orbiviruses are pathogenic for animals; *African horse sickness virus*, equids manifest in three ways dunkop, dikkop and mixed form with 90% death rate. *Kasba virus* causes Chuzan disease in calves, *Bluetongue virus* responsible for “bluetongue or ovine catarrhal fever” or dancing disease in domestic and wild ruminants and *Epizootic hemorrhagic disease virus* also infect wild ruminants. The *Ibaraki virus* show symptoms like bluetongue-like in cattle, *Equine encephalosis virus* similar to *American horse sickness virus* in equine, *Peruvian horse sickness virus* with neurological disorders in horse up to 80% mortality and *Yunnan virus* also causes neurological disorders in donkey, cattle, sheep, and dog (Mellor and Hamblin, 2004; Kedmi *et al.*, 2010; Maclachlan and Guthrie, 2010; Kim *et al.*, 2011; Maclachlan, 2011; Cowled *et al.*, 2012).

1.2.71. Epidemiology:

Orbiviruses distributed worldwide (Hubálek *et al.*, 2014).

1.2.72. Treatment:

None (Hubálek *et al.*, 2014).

1.2.73. Vaccine:

Vaccines are available against two notable orbiviruses *African swine fever virus* and *Bluetongue virus* (Paton and Taylor, 2011).

1.2.74. Genus *Seadornavirus*; History and taxonomy:

Genus contains only three viruses (Table 14).

Table 14. Summary of genus *Seadornavirus*.

Serogroup	Species	Strains	Tentative	Total	Ticks	Mosq	Sandflies	N D	Flu-like	Ence	N/K
BANV	1	-	-	1	-	1	-	-	-	1	-
KDV	1	-	-	1	-	1	-	-	-	-	-
LNV	1	-	-	1	-	1	-	-	-	1	-

1.2.75. Arthropod vectors:

All three viruses transmitted by mosquitoes (Table 14).

1.2.76. Human disease:

Only Banna virus is causes encephalitis in human and other two viruses are non-pathogenic (Table 14)

1.2.77. Animal disease:

The only *seadornaviruses* isolated from human (Attoui *et al.*, 2005).

1.2.78. Epidemiology:

The *Seadornaviruses* are only endemic in Southeast Asia (Attoui *et al.*, 2005).

1.2.79. Treatment:

No specific treatment except relief of fever and pain (Attoui *et al.*, 2005).

1.2.80. Vaccine:

None (Attoui *et al.*, 2005).

1.2.81. Family Rhabdoviridae; History and classification

This family are currently composed of eleven genera based on antigenicity, phylogenetic analysis and structural properties (Bodewes *et al.*, 2014). Eight that infect vertebrates (Ephemerovirus, Lyssavirus, Novirhabdovirus, Perhabdovirus, Sprivivirus, Tibrovirus, Tupavirus and Vesiculovirus), two genera responsible for plants (Cytrorhabdovirus and Nucleorhabdovirus) and Sigmavirus found in invertebrates. Three of them genera are containing arthropods-borne agents Ephemerovirus, Tibrovirus and Vesiculovirus ((Table 15).

Table 15. Summary of family *Rhabdoviridae*.

Genus	Mode of transmission	Infect	Reference
Cytorhabdovirus	Insect: Planthopper	Plants	(Jackson <i>et al.</i> , 2005)
Ephemeroviruses	Arthropods	Cattle	(Blasdell <i>et al.</i> , 2014)
Lyssaviruses	Bite by a rabid mammals	Humans	(Hicks <i>et al.</i> , 2012)
Novirhabdovirus	Waterborne	Fish	(Reichert <i>et al.</i> , 2013)
Nucleorhabdovirus	Insect: Leafhopper	Plants	(Jackson <i>et al.</i> , 2005)
Perhabdovirus	Waterborne	Fish	(Gadd <i>et al.</i> , 2013)
Sigma Virus	Vertically through eggs and sperm	Drosophila spp.	(Longdon <i>et al.</i> , 2010)
Sprivirus	Waterborne	Fish	(Ghasemi <i>et al.</i> , 2014)
Tibrovirus	Arthropods	Cattle	(Gubala <i>et al.</i> , 2011)
Tupavirus	?	Mammals	(Allison <i>et al.</i> , 2011)
Vesiculovirus	Arthropods	Livestock and Humans	(Letchworth <i>et al.</i> , 1999)
Unassigned	-	-	--

1.2.82. Genus *Ephemerovirus*; History and taxonomy

The origin of Bovine Ephemero Fever virus was first noticed as an infectious agent in East Africa in mid-nineteenth century (Nandi and Negi, 1999). The genus Ephemerovirus is one of eleven currently recognized genera in the family Rhabdoviridae. This genus divided into two serogroups with eight viruses, The BEFV group contains six virus five species and one tentative. Two viruses represented from second group MALV (Table 16).

Table 16. Summary of genus *Ephemerovirus*.

Serogroup	Species	Strain	Tentative	Total	Ticks	Mosq	Sandflies	S+M	ND	Flu-like	Enc	N/K
BEFV	5	-	1	6	-	2	1	1	2	1	-	5
MALV	-	-	2	2	-	1	-	1	-	-	-	2
Total	5	0	3	8	-	3	1	2	2	1	0	7

1.2.83. Arthropod vectors:

Principal vectors are mosquitoes for the genus *Ephemerovirus*. Mosquitoes transmitted virus two from BEFV group and one from MALV group. However, biting Sandflies also additional vector in BEFV group. Two vectors one from each group (Mosquitoes and sandflies) and two vectors still unknown from BEFV group (Table 16).

1.2.84. Vertebrate hosts

The ruminants are reservoir host (Hubálek *et al.*, 2014).

1.2.85. Human disease

Viruses belong to this genus not pathogenic for humans (Table 16).

1.2.86. Animal disease

The Bovine ephemeral fever virus causes three days sickness in cattle and water buffaloes, dengue fever of cattle, stick sickness and bovine epizootic fever and second virus from this genus Kotonkan virus manifest an ephemeral fever-like illness in cattle (Tomori *et al.*, 1974; Doherty, 1977; Nandi and Negi, 1999).

1.2.87. Epidemiology:

Bovine ephemeral fever is present throughout Africa, Middle East, northern Australia and Southeast Asia. Other virus from this genus Kotonkan distribute in Nigeria (Africa) (Hubálek *et al.*, 2014).

1.2.88. Treatment

There is not any specific treatment available.

1.2.89. Vaccine:

Only an attenuated vaccine has been using in Australia and Japan against BEFV within genus (Uren *et al.*, 1994).

1.2.90. Genus *Tibrovirus*; History and taxonomy

This genus is name as an adapted from the species Tibrogargan (TIBV) and it was first isolated in 1976 from a pool of sandflies in Australia (Queensland). The genus *Tibrovirus* newly recognized with two species, Tibrogargan virus (TIBV) and Coastal Plains virus (CPV) (Lauck *et al.*, 2015) (Table 17 and sup Table 13).

Table 17. Summary of genus *Tibrovirus*.

Serogroup	Species	Strain	Tentative	Total	Ticks	Mosq	Sandflies	S+M	ND	Flu-like	Enc	N/K
TIBV	2	-	-	2	-	-	2	-	-	-	-	2
Total	2	-	-	2	0	0	0	0	0	0	0	2

1.2.91. Arthropod vectors:

Usually, both viruses are transmitted via biting of sandflies (TableXX).

1.2.92. Vertebrate hosts:

Bovine and water buffalos are vertebrate host (Gubala *et al.*, 2011).

1.2.93. Human disease:

None of responsible for disease in human (Lauck *et al.*, 2015).

1.2.94. Animal disease:

Not reported (Gubala *et al.*, 2011).

1.2.95. Epidemiology:

Initially, Tibroviruses presence throughout Australia and New Guinea (Cybinski and Gard, 1986).

1.2.96. Treatment

Not required.

1.2.97. Vaccine:

No.

1.2.98. Genus *Vesiculovirus*; History and taxonomy

In 1916, first Vesiculovirus or Vesicular Stomatitis Virus (VSV) was reported in United States an epidemic in livestock (Hanson, 1952). The genus Vesiculovirus have total 27 viruses, nine of them are confirmed species and eighteen viruses are tentative (Table 18 and sup Table 14).

Table 18. Summary of genus *Vesiculovirus*.

Serogroup	Species	Strain	Tentative	Total	Ticks	Mosq	Sandflies	M+S	N D	Flu- like	Enc	N/ K
Boteke	-	-	1	1	-	1	-	-	-	-	-	1
Kwatta	-	-	1	1	-	1	-	-	-	-	-	1
VSIV	2	-	-	2	-	-	1	1	-	2	-	-
VSIJ	1	-	-	1	-	-	-	1	-	1	-	-
VSV	6	-	12	18	-	8	6		4	3	-	15
Ungrouped	-	-	4	4	-	4	-	-	-	-	-	4
Total	9		18	27	-	14	7	2	4	6	-	21

1.2.99. Arthropod vectors:

Mosquitoes are main vector link with 14 viruses, seven viruses by Sandflies, four viruses are through both sandflies and mosquitoes and four viruses are transmitted with unknown vectors.

1.2.100. Vertebrate hosts:

There are several reservoir host like; Cattle, horse, pig, deer, rodents and bats (Hubálek *et al.*, 2014).

1.2.101. Human disease:

A total six viruses causes flu-like symptoms in humans and other 21 viruses non-pathogenic for human (Table 18).

1.2.102. Animal disease

Four members of Vesiculovirus clinical disease presents in Livestock. The Cocal virus (Indiana-2), Vesicular stomatitis- New Jersey virus and Vesicular stomatitis- Indiana virus causes vesicular stomatitis. Vesicular stomatitis Alagoas virus (Indiana-3) are pathogenic for cattle, equids cause tongue vesicles and fever causes (Letchworth *et al.*, 1999).

1.2.103. Epidemiology

Vesiculoviruses are present only in the Americas especially south Mexico, Central America and some regions of South America (Hubálek *et al.*, 2014).

1.2.104. Treatment

No specific treatment still available against Vesiculoviruses (Jadi *et al.*, 2011).

1.2.105. Vaccine

No vaccine (Jadi *et al.*, 2011).

1.2.106. Family *Togaviridae*; History and classification

This family name come from the Roman Cloak (in Latin = toga) for this reason called togaviruses. The family togaviridae is categorized into two genera: Alphavirus and Rubivirus. The Rubivirus genus is containing of a single member its Rubella virus (also called German measles) mode of transmission by droplet spread or direct contacts (Jose *et al.*, 2009; Mangala Prasad *et al.*, 2013).

1.2.107. *Alphavirus* genus; History and taxonomy

In 1930, first Alphavirus was isolated as Western Equine Encephalitis virus by Dr. Karl Friedrich Meyer with very interesting story. During encephalitis in horse at San Joaquin Valley (California, USA), Scientist team tried to get head of horse symptomatic for encephalitis and owner of horse threatened team “ I won’t sell the horse, and you try to do anything to the horse I shoot you”. But, Dr. Meyer deal with former wife when he was asleep in exchange horse head for USD 20, the team successfully isolated virus from the brain tissue (Sabin, 1980). The genus Alphavirus generally referred to; the viruses responsible for rheumatic disease in humans (Old World) and viruses especially associated with encephalitis disease in the Americas (New World) (Suhrbier *et al.*, 2012). The *Alphavirus* genus consists of about 36 different viruses divided antigenically into seven serogroups and some viruses are still unclassified they not have their yet confirm serogroup status (Table 19).

Table 19. Summary of genus *Alphavirus*.

Serogroup	Species	Strains	Tentative	Total	Ticks	Mosq	Sandflies	M+S	ND	Flu-like	Enc	R.A	N/K
BFV	1	-	-	1	-	1	-	-	-	1	-	-	-
EEEV	2	-	-	2	-	1	-	-	1	-	1	-	1
MIDV	1	-	-	1	-	1	-	-	-	-	-	-	1
NDUV	1	-	-	1	-	1	-	-	-	-	-	-	1
SFV	9	1	-	10	-	9	-	-	1	-	1	5	4
VEEV	9	-	-	9	-	9	-	-	-	2	3	-	4
WEEV	8	3	-	11	-	9	-	-	2	-	1	2	8
Ungrouped	-	-	1	1	-	-	-	-	1	-	-	-	1
Total	31	4	1	36	0	31	0	0	5	3	6	7	20

1.2.108. Arthropod vectors:

The mosquitoes are main vector for the genus *Alphavirus* with approximately 31 viruses and 5 viruses are transmitted through unknown vectors (Table 19).

1.2.109. Vertebrate reservoir hosts:

The vertebrate reservoir hosts are generally mammals, birds and primates (Weaver and Barrett, 2004).

1.2.110. Human disease:

Twenties viruses are non-pathogenic for humans from all serogroups except Barmah forest (BFV) virus. A total sixteen viruses are able to infect humans and causes flu-like to encephalitis except from three complexes Middleburg virus (MID), Ndumu virus (NDU) and one from ungrouped. Seven viruses are causes Rash and Arthritis (R.A), five viruses are from Semeliki forest (SF) and two from Western Equine Encephalitis virus (WEEV). Six viruses are responsible for encephalitis, one from EEEV, one from SF, also one from WEEV and three viruses are from VEEV. About flu-like, one virus from (SF) and two viruses are from VEEV (Table 19).

1.2.111. Animal disease:

There are nine exotic Alphaviruses significant on health of animals, the members within the sero-complex Western equine encephalitis virus is contains four viruses; Buggy Creek virus has little impact on house sparrows (O'brien and Brown, 2011), Highland J virus cause encephalitis in horses and recognized pathogenic for various avian species (Weaver *et al.*, 1997). Sindbis virus causes encephalitis in horses and notable virus in this group Western Equine Encephalitis virus encephalomyelitis (Calisher *et al.*, 1986). One viruses from Eastern Equine Encephalitis (EEEV) serogroup and one Venezuelan Equine Encephalitis (VEEV) causes encephalitis in equines (Zacks and Paessler, 2010). From Semiliki forest virus group; two viruses Getah virus pathogenic for racehorse and causes fever and other semiliki forest virus responsible for encephalitis in horses (Robin *et al.*, 1974; Fukunaga *et al.*, 2000) . One virus from Middelburg serocomplex (Middelburg virus) also responsible for encephalitis in equines (Attoui *et al.*, 2007).

1.2.112. Epidemiology:

Alphaviruses are dispersed globally (Go, Y. Y.2014).

1.2.113. Treatment:

Currently, there are no licensed specific treatment available except symptoms supportive drugs for any Alphavirus (Phillips *et al.*, 2013).

1.2.114. Vaccine:

We are still waiting licensed vaccines for humans use, but some (EEEV, VEEV and WEEV) unlicensed vaccines are available to protect at-high risk laboratory personnel (Hoke, 2005). For the veterinary use, vaccines (EEEV, VEEV and WEEV) are available for routine use to control infection in endemic areas (Barber *et al.*, 1978).

1.2.115. Family *Asfarviridae*; History and classification:

In 1910, R.E Montgomery discovered first African swine fever virus (African swine fever and related viruses) from an ill pig in Kenya (at Kabete, Kiambu County). It was observed that the occurrence of cases appear when domestic pigs come into close

contact with wildlife species, specially warthogs (Costard *et al.*, 2009). There is a unique genus Asfarvirus in family *Asfarviridae*.

1.2.116. Genus *Asfarvirus*: History and taxonomy

This genus has only one species and its called African swine fever virus (Table 20).

Table 20. Summary of genus *Asfarvirus*.

Serogroup	Species	Strains	Tentative	Total	Ticks	Mosq	Sandflies	ND	Flu-like	Ence	R.A	N/K
ASF	1	-	-	1	1	-	-	-	-	-	-	1
Total	1	0	0	1	1	0	0	0	0	0	0	1

1.2.117. Arthropod vectors:

Ticks are main arthropods vector (Hess, 1986).

1.2.118. Vertebrate hosts:

The warthogs are reservoir host for ASFV (Thomson, 1985; Hess, 1986)

1.2.119. Human disease:

Not reported (Loh *et al.*, 2009).

1.2.120. Animal disease

Hog cholera (classical swine fever) in swine and wild boar (Schlafer and Mebus, 1984).

1.2.121. Epidemiology:

Worldwide (Costard, S.2009).

1.2.122. Treatment:

No treatment for ASF (Zaberezhnyĭ *et al.*, 2012)

1.2.123. Vaccine

There is no effective vaccine available for African swine fever (Penrith *et al.*, 2013).

1.2.124. Genome of Roboviruses and Arboviruses

All these viral families have a RNA genome except of the family *Asfarviridae* that contains DNA (Table 21). Therefore, RNA viruses replication include high mutation and short replication times in compare to DNA viruses. RNA viruses are able to jump from its original host into a new host species and adapt to changing new environmental conditions.

Table 21. Genome of roboviruses and arboviruses

Viral Family	Enveloped	Sence	Segments	Reference
<i>Arenaviridae</i>	Yes	ssRNA (-)	Bisegmented (S and L)	(Buchmeier Mj, 2007)
<i>Asfarviridae</i>	Yes	dsDNA	Non-segemnted	(Dixon <i>et al.</i> , 2013)
<i>Bunyaviridae</i>	Yes	ssRNA (-)	Tripartile (S, M and L)	(Nichol St, 2005)
<i>Flaviviridae</i>	Yes	ssRNA (+)	Non-segmented	(Mukhopadhyay <i>et al.</i> , 2005)
<i>Orthomixoviridae</i>	Yes	ssRNA (-)	Six to Eight segmented	(Kawaoka Y., 2005)
<i>Reoviridae</i>	No	dsRNA	Ten - twelve (size S,M and L)	(Verwoerd <i>et al.</i> , 1972)
<i>Rhabdoviridae</i>	Yes	ssRNA (-)	Non-segmented	(Lee <i>et al.</i> , 2014)
<i>Togaviridae</i>	Yes	ssRNA (+)	Non-segmented	(Cheng <i>et al.</i> , 1995)

1.2.125. Diagnosis of viral infections:

Historically, the diagnosis of viral infections includes virus isolation in cell culture, the direct immunofluorescence and enzyme immunoassays methods. Recently, the polymerase chain reaction (PCR) has revolutionized the diagnosis of viral infections by increasing the detection sensitivity and enabling the detection of several viruses in parallel. However, the multiple steps necessary for performing conventional PCR make

it too laborious and prone of contamination with the amplification product, generating false positives results. This problem was solved with the development of the real-time PCR. This method is based on DNA amplification in addition to a fluorescent agent that is detected in the same thermal cyclers that the amplification occurs. This method requires no manipulation after amplification and is therefore called a closed system. The main advantages of this homogeneous system are: reducing the time required for the test the possibility of viral load and low risk of contamination with the amplified product (Böhland, 1992). However, the technology currently available for real-time PCR allows detection of only a few viruses in a single test. To solve this limitation, DNA microarray platform are being developed for the detection of several viral agents (Palacios *et al.*, 2007). This method consists of detecting the viral genome by means of DNA probes, which are fixed on silica plates. The microarray platform has the potential to detect multiple viral agents. The number of viral agents that can be detected with the microarray platform far exceeds any other method currently available hundreds of thousands of probes can be printed on a sheet of 70 x 20 mm to detect a large number of viral agents.

2. JUSTIFICATION

Several viruses transmitted by rodents and arthropods cause permanent threat to human health; in addition, others new viruses could become emergent viruses. Therefore, there is a great interest in the development of method for early diagnosis of infection with these viruses. Traditional diagnostic methods can detect only one or few viruses in a single assay, turning the final diagnosis very slow and expensive when several assays are needed. The DNA microarray platform has emerged recently as a high-throughput method that can be use to screen up to thousand of pathogens in a single assay, providing a early and cheaper final diagnosis.

3. OBJECTIVES

3.1. GENERAL:

To develop a DNA microarray platform for the detection of viruses transmitted by rodents and arthropods

3.2. SPECIFIC:

- 1) To develop a DNA microarray slide (RoboArboVirusChip) to target viruses from the *Arenaviridae*, *Asfarviridae*, *Bunyaviridae*, *Flaviviridae*, *Reoviridae*, *Rhabdoviridae* and *Togaviridae* families.
- 2) To prepare a control DNA microarray slide by using published probes.
- 3) To evaluate the RoboArboVirusChip and control slides by using laboratory reference viruses.
- 4) To determine the lower limit of detection of the RoboArboVirusChip.
- 5) To evaluate the applicability of the RoboArboVirusChip for detecting viruses in serum samples.

Figure 1. WORK PLAN.

4. MATERIAL AND METHODS

4.1. Viral reference Strains:

The viral reference strains were available in a repository at the Center for Research in Virology (CPV), Faculty of Medicine of Ribeirao Preto (FMRP), University of Sao Paulo. A total 21 known viruses were used in this study (Table 22).

Table 22. Reference viruses used in this study.

Family	Genus	Virus	Abbreviation	Strain	Cell	BSL
<i>Bunyaviridae</i>	<i>Orthobunyavirus</i>	<i>Guaroa virus</i>	GROV	BeAn 277	C6/36	2
		<i>Oropouche virus</i>	OROV	BeAn19991	C6/36	3
	<i>Hantavirus</i>	<i>Rio Mamore virus</i>	RIOMV	OM 556	VERO E6	3
<i>Flaviviridae</i>	<i>Flavivirus</i>	<i>Bussuquara virus</i>	BSQV	BeAn 4073	C6/36	2
		<i>Dengue virus type -1</i>	DENV-1	Mochizuki	C6/36	2
		<i>Dengue virus type -2</i>	DENV-2	New Guinea C	C6/36	2
		<i>Dengue virus type -3</i>	DENV-3	H87	C6/36	2
		<i>Dengue virus type -4</i>	DENV-4	H 241	C6/36	2
		<i>Iguape virus</i>	IGUV	Span 71 686	C6/36	2
		<i>Ilheus virus</i>	ILHV	BeH 7445	C6/36	2
		<i>Rocio virus</i>	ROCV	SPH 34675	C6/36	3
		<i>St.Louis encephalitis virus</i>	SLEV	BeH 356964	C6/36	3
		<i>West Nile virus</i>	WNV	NY99-4132	C6/36	3
		<i>Yellow fever virus</i>	YFV	17D (vaccine)	C6/36	3
<i>Rhabdoviridae</i>	<i>Vesiculovirus</i>	<i>Piry virus</i>	PIRYV	BeAn24232	C6/36	3
<i>Togaviridae</i>	<i>Alphavirus</i>	<i>Mayaro</i>	MAYV	BeAn243	C6/36	2

		<i>virus</i>				
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4.2. Design of the DNA microarray slide for viruses transmitted by rodents and arthropods (RoboArboVirusChip)

4.3. Database

Roboviruses and arboviruses were selected from the virus taxonomy list of the International Committee on the taxonomy of viruses (ICTV 2011 Master Species List (MSL) released in February 2012 (E.J. San Diego, 2012) and from the Arbovirus Catalog-Virus section (Arbocat) (<https://wwwn.cdc.gov/arbocat/Virusbrowser.aspx>). All sequences of roboviruses and arboviruses available in the GenBank were retrieved for probes design. The obtained sequences belong to viruses within seven families and 13 genera: Family *Bunyaviridae*: *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* and *Hantavirus* genera; family *Flaviviridae*: *Flavivirus* genus; family *Reoviridae*: *Orbivirus*, *Coltivirus* and *Seadornavirus* genera; family *Rhabdoviridae*: *Vesiculovirus*, *Ephemerovirus* genera; *Togaviridae*: *Alphavirus* genus; family: *Asfarviridae*, *Asfarvirus* genus; and family: *Arenaviridae*, *Arenavirus* genus. We have found 412 viral species, tentative or strains with sequences available in GenBank and we obtained total 2144 viral sequences from viruses mentioned above (Table 23).

Table 23. Summary of viruses and sequences retrieved from GenBank.

Family	Genus	Vector Arthropods/ Rodents	Number of viruses	Number of Sequences	Number of specific probes	Number of generic probes
<i>Bunyaviridae</i>	<i>Orthobunyavirus</i>	Mosquitoes, ticks and flies Culicoides	86	272	690	5
	<i>Nairovirus</i>	Ticks, flies, mosquitoes culicidae	14	41	100	6
	<i>Phlebovirus</i>	Sandflies, mosquitoes and ticks	56	262	627	ND
	<i>Hantavirus</i>	Rodents	73	299	638	18
<i>Flaviviridae</i>	<i>Flavivirus</i>	Mosquitoes and ticks	74	165	418	13
<i>Reoviridae</i>	<i>Orbivirus</i>	Sand flies, mosquitoes and ticks	18	434	783	6
	<i>Coltivirus</i>	Ticks	2	12	41	ND
	<i>Seadornavirus</i>	Mosquitos	2	20	83	ND
<i>Rhabdoviridae</i>	<i>Vesiculovirus</i>	Sandflies and mosquitoes	18	20	122	11
	<i>Ephemerovirus</i>	Sandflies	5	7	57	ND
<i>Togaviridae</i>	<i>Alphavirus</i>	Mosquitos	29	386	130	8
<i>Orthomixoviridae</i>	<i>Thogotovirus</i>	Ticks	ND	ND	ND	ND
<i>Asfarviridae</i>	<i>Asfivirus</i>	Culicoides	1	100	17	ND

<i>Arenaviridae</i>	<i>Arenavirus</i>	Rodents	41	126	503	20
Total	14	----	419	2144	4209	87

ND: Not done

4.4. Oligonucleotide Probe design

For selection of specific probes, the relevant open reading frame of viral sequences from multiple genes including a highly conserved region corresponding to each viral species were aligned using the program CLC Main Workbench (CLCbio, USA). Alignment was analyzed using the CLC Main Workbench or SCPrimer (www.scpriimer.cpmc.columbia.edu) programs. The suggested 60-mer probes were selected based on those having melting temperature 60 -77°C, higher G/C pair content 40 to 60%. For viruses with a single genome segment, up to 10 probes were selected; for those with more than one genome segment, up to three probes per segment were selected. For family *Reoviridae*, *Orbivirus* genus contains 10 segments; *Coltivirus* and *Seadornavirus* contain 12 segments. For these viruses, the probes were designed for VP2 (S-2), VP5 (S-6) and NS3 (S-10) segments. The sequences of the probes obtained were analyzed with the BLAST program (<http://www.ncbi.nlm.nih.gov/>) and those with >95% identity with the virus in question was selected.

For selection of generic probes, sequences of representative viruses within each genus were aligned. These alignments were used to select generic 20-35-mer probes from conserved genomic regions as mentioned above.

4.5. Preparation of RoboArboVirusChip

The slide was drawn using the Agilent eArray program (<https://Earray.Chem.Agilent.Com/Earray/>). The slide was designed in such a manner that Agilent added the necessary repetitions of each probe together with the positive and negative controls sequences by default to reach 15,000 probes per array. A slide containing eight arrays with 15,000 probes were designed.

4.6. Control slide (GreeneChipVr):

The control slides were used for compare reference viruses with RoboArboVirusChip. The GreeneChipVr design for all viruses causes problem in vertebrates by Dr. W. Ian Lipkin group at the Mailman School of Public Health at Columbia University, New York-USA. The probes of the GreeneChipVr are available on the Gene Expression Omnibus (GEO) database with GEO number GPL10319 were used. The virus species corresponding to each probe is not indicated. For the identification of viral species, each probe was checked by BLAST. To prepare this slide in the same format of eight arrays with 15,000 probes/array, probes corresponding to various controls were removed. Remaining mostly, those corresponding to the virus.

4.7. Preparation of viral stocks

All viral infections were performed by using C6/36 or Vero E6 cell line, which were cultured in 5 ml of medium Leibowitz L15. The C6/36 cells were grown 24 hours before infection in 25 cm² flask and Vero E6 cells were in 25cm² tissue culture flask (vented cap) with 10% inactivated fetal calf serum (FCS), 10% tryptose phosphate broth (Oxid, England), 100 U/ml penicillin and 100 µg/ml streptomycin. The plate kept at 28°C for C6/36 cells and for Vero E6 kept in a humidified incubator at 30°C with 5% CO₂. The culture medium was aspirated from a bottle and the cell monolayer was 3 times washed with 1x phosphate buffer solution (1X PBS), in order to remove dead cells and traces of fetal calf serum, After the cells became confluent, a monolayer was infected with 10 µl of viral suspension then, the bottle was shaken every 15 minutes and incubated for 1 hour at 37°C so that the virus was absorbed. Then L-15 medium with 2% FCS was added and the cells were incubated at 37°C. Viral infection allowed proceeding until the onset of cytopathic effect (CPE). The supernatant was collected for further use such as RNA extraction, RT-PCR, Real- time PCR, Viral titration and microarray experiments. The cells were harvested, viral infection was confirmed by RT-PCR and indirect immunofluorescence, the supernatants were centrifuged at 3,000 rpm for 5 minutes, supplemented with 20% FCS aliquot in to sterile tubes (200µl) and stored at -80°C until use.

4.8. Confirmation of viral infection by indirect immunofluorescence

The viral infected and normal cells (C6/36 or Vero E6) were harvested and cells washed 3 times with 1XPBS by centrifugation at 1000 rpm for 10 minutes at 4°C, then cells were re-suspend in 1XPBS and 20µl was dispensed onto immunofluorescent glass slides and allowed to air-dry at room temperature. Cells were fixed onto a slide and place in a coplin jar for 10 minutes containing 100% cold acetone, then the cells were permeabilized to rupture cell wall in 0.2% Triton X-100 and 1% FCS for 5 minutes in ice, then the cells were washed three times with 1X PBS containing 1% FCS for 10 minutes. Fixed cells were incubated with 20µl of a 1:100 dilution of primary antibody Mouse immune ascetic fluids (MIAF diluted in 3xPBS containing 3% FCS) at 37°C in a humidified chamber for 1 hour to specifically binds the target molecule and then the cells were washed 3 times with 1X PBS containing 1% FCS for 10 minutes. Thereafter, added 20µl of a 1/256 dilution of the secondary antibody which carries the fluorophore (anti-mouse IgG raised in rabbit were added, in conjunction with FITC), containing Evans blue dye (1/20.000, diluted in blocking buffer) and the slide was incubated at 37°C in humidified chamber for 1 hour and again cells washed four times with 1XPBS for 10 minutes. Finally, mounted slides were visualized under a fluorescence microscope.

4.9. Confirmation of viral infection by RT-PCR

4.9.1. Viral RNA extraction

RNA from virally infected and uninfected cell cultures was purified by using AxyPrep viral RNA/DNA Miniprep Kit (Axigen, USA), following the protocol recommended by the manufacturer. The viral RNA was suspended in 60µl of elution buffer.

4.9.2. The cDNA synthesis

The reaction mixture for cDNA synthesis had a total volume 20µl, contained 10µl of RNA, 15ng/µl random primer (Invitrogen USA), 0.5mM dNTPs (Invitrogen USA) and added 1 drop of oil. The mixture was incubated at 95°C for 1 minute and 20 seconds and quickly cooled in ice for 5 minutes. Into this mixture, was added 4 mM DTT, 4U RNase inhibitors (RNaseOUT, Invitrogen USA), 200U Reverse Transcriptase MMLV (Invitrogen, USA) and 4µl of 5X buffer (250mM Tris-HCL [pH 8,3], 375mM

KCl, 15mM MgCl₂). Mixture was incubated at 25°C for 10 minutes; second incubation cycle was performed at 37°C for 1 hour and 85°C for 5 minutes in a Thermo cycler (BIO-RAD, USA). The cDNA was treated with 1U RNase H at 37°C for 30 minutes and stored at -20°C until use.

4.10. The reverse-transcription PCR amplification

The final volume of the PCR reaction mixture was 50µl, contained: 4µl cDNA, 0.2 mM dNTPs, 0.3 µM primer sense and complementary for (Bronzoni et al., 2005), (Ref of Piry, Oropauche and Iguape) 1.5 U enzyme Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA), 10X buffer (600mM Tris-SO₄ [pH 8.9], 180mM NH₄ SO₄) suitable for each enzyme and 2 mM Mg₂SO₄. Amplification was performed using thermo cycler MyCycler (BIO-RAD, USA). The reaction mixture was heated at 94°C for 2 minutes and the temperature profile for PCR was as follow:

94°C for 30 seconds	Initial denaturation	
56°C for 01 minute	Denaturation	45 cycles
68°C for 05 minutes	Annealing & Extension	

the reaction ending with a final extension at 68°C for 7 minutes.

4.11. Visualization of DNA:

An aliquot of 10µl of the amplification products were evaluated by electrophoresis, loaded on 1.8% agarose gel (Pronadisa, Spain) in 40 mM Tris-Acetate-1mM EDTA (TAE) buffer. The bands of DNA were achieved through gel stained with GelRed and visualized with UV light.

4.12. Determination of the viral infectivity titer by plaque assay

A total 15 viruses were titrated by Plaque assay (Burleson, 1992). VERO E6 cells were washed 3 times with sterile 1X PBS and trypsinized, seeded cells were grown in 24-well plate (2x10⁵ cells/wells) in L-15 medium supplemented with 10% FBS and plates were incubated at 28°C with 5% CO₂ in an incubator. For each dilution, 2 ml sterile tubes were used with decade dilutions from 1⁻¹ to 10⁻¹⁰ and added to 900µl of L-15 medium without FCS. One hundred µl of the viral supernatant were added to the first

vortex tube. Plates were checked under a light microscope to confirm >70% confluence and homogenous cell distribution, medium was aspirated from the cells with a sterile Pasteur pipette without disturbing the cells by vacuum. The cells were infected with 400µl/well of 10-fold serially diluted virus in duplicate, 400µl of L-15 medium was added without FCS as a negative control and 100µl of viral supernatant was used as positive control and incubated 1 hour at 28°C with 5% CO₂ in an incubator. After an incubation period, supernatant was carefully aspirated out from the cells with a sterile Pasteur pipette; the monolayers were covered with a nutrient medium containing agar. It One thousand µl of carboxymethyl-cellulose (CMC) was added to each well. This solution was prepared with 1.8% of CMC in 2X L-15 medium containing 2% of FCS and 1% of antibiotic (Ab) and the cells were incubated as per virus plaque forming character 3 to 10 days post infection at 28°C with 5% CO₂ in an incubator. The medium was removed and the cells were fixed and stained by addition of 200µl of black naphthalene dye that remained in contact with the cells for 40 minutes at room temperature in a mixer. The dye was aspirated and the cells were dried at room temperature. The lysed plates were quantified by viewing under a stereoscopic microscope and the light areas of cell foci were counted to calculate the viral titer expressed as plaque forming units per ml (PFU/ml).

4.13. *Dengue virus* RNA detection and quantification: Quantitative real-time RT-PCR (qRT-PCR)

Viral RNA detection was carried out by real-time RT-PCR using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, USA), as previously described (Dos Santos *et al.*, 2008). Briefly, the 25-µL reaction mixture contained 0.5 µL of SuperScript III RT Platinum Taq Mix, 0.2 mM of each primer, 12.5 µL of 2X SYBR Green, and 5 µL of purified RNA. The amplification program was as follows: 50°C for 20 min for reverse transcription, 95°C for 5 min for reverse transcriptase inhibition and *Taq* DNA polymerase activation, followed by 45 cycles of PCR amplification with denaturation at 95°C for 15 sec, annealing at 60°C for 40 sec, and extension at 72°C for 30 sec. Finally, to verify the specificity of the PCR products, a melting curve was

constructed by incubating the amplification products from 60°C to 90°C with an increase of 0.2°C/sec. The melting temperature (T_m) values of the specific amplicons were in the range of 80.57°C-81.73°C. For viral load determination, a standard curve was constructed using RNA transcribed *in vitro* from a plasmidial clone containing a fragment of 2500 base pairs (bp) corresponding to the 5' end of DENV-3 strain D3BR/RP1/2003 (Aquino *et al.*, 2006). This plasmid was prepared as follows: the 2500-bp fragment was amplified by RT-PCR and inserted into the plasmid pXL (Invitrogen, USA), which was used to transform *Escherichia coli*. An aliquot of 250 μ L of the bacteria was inoculated into 10 mL of LB+ampicillin medium, followed by incubation at 37°C for 14 h with shaking. The plasmid was purified using the QIAGEN Plasmid Mini Kit (Qiagen, Germany) following the manufacturer's recommendations. The plasmid was linearized by digestion with Bam HI, subjected to electrophoresis through a 1% agarose gel, and purified from the gel using the QIAquick Gel Extraction Kit (Qiagen, Germany) following the manufacturer's specifications. For RNA preparation, 1 μ g of the linearized plasmid was transcribed using RNAMaxx High Yield Transcription Kit (Stratagene, USA) following the manufacturer's specifications. The cDNA was digested using RQ DNase (Promega, USA) at 37°C for 20 min. After this, the RNA was purified using the QIAamp Viral RNA Kit (QIAGEN, Germany), and the concentration was determined by spectrophotometry at 260 nm. Based on the concentration and size of the RNA product (2500 bp), the copy number equivalent to the genomic RNA was calculated. Serial decimal dilutions of this RNA were prepared to construct the standard curve by real-time RT-PCR for determination of the virus titer, which was expressed in copy numbers per milliliters.

4.14. Serum samples

The human serum samples were used for the determination of Dengue virus in this study from serum bank at our virology laboratory called “Arbovirus”. All these samples were collected during year 2006-2014 from deferent primary health care centers at Ribierao Preto.

4.15. Microarray experiment

2.9.1. Positive RNA Spike controls

The Agilent RNA spike positive controls are optimized to anneal to complementary probes on the microarray with minimal self-hybridization or cross-hybridization. The Spike-In probe sets were designed not to have complementary sequences to the biological samples used on most microarrays, even to the viruses included in our array. The RNA spike control was diluted 1:5000 for use in the experiment.

4.16. cDNA synthesis (Reverse Transcription):-

The synthesis of cDNA was performed using a random primer eight nucleotides linked to an artificial specific sequence (SIA, 5 'TAG GTC CAG TCC GTT TCN NNN NNN N3') (Bohlander et al., 1992). The reaction mixture for synthesis of cDNA (total volume of 25µl) contained: 12 µl of viral RNA, 1.2 mM CIA random primers (Invitrogen, USA), 2 µl of 1:5000 dilution of Spike (Agilent, USA) , 0.4 mM dNTPs (Invitrogen, USA), 40 U RNase inhibitor (RNaseOUT, Invitrogen, U.S.), 4 mM DTT, 200 U of M-MLV reverse transcriptase (Invitrogen,USA) and 5 µl of 5X buffer (250mM Tris-HCl [pH 8.3], 375mM KCl, 15mM MgCl 2). The mixture was incubated at 25°C for 10 minutes followed by incubation at 37°C for 2 hours and finally at 85°C for 5 minutes.

4.17. The polymerase chain reaction (PCR) amplification

The PCR mixture contained in a ratio of 1:9 primers SIAN (5 'GTA GCA CGT TTC CCA GGT CTC 3'), the latter recognizes a specific region of primer SIA. The PCR reaction mix had a final volume of 50µl, containing 4 µl of cDNA, 2 mM MgCl₂, 0.2 mM dNTP, 5U enzyme Platinum Taq DNA Polymerase (Invitrogen, USA), 5µl of 10X

buffer (SO₄ 600mM Tris [pH 8.9], NH₄ SO₄ 180mM) suitable for each enzyme. Amplification was performed using Thermo Cycler MyCycler (BIO-RAD, USA). The reaction mixture was submitted to 8 cycles of 94°C for 30 seconds, 25°C for 1 min and 72°C for 1 minute, followed by 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C extension for 1 minute.

4.18. Purification of PCR product

PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer's recommendations. The PCR product was eluted with 15µl of RNase /DNase free water, before proceeding with the labeling reaction.

4.19. Addition of the poly -T tail:

The PCR products were attached to a poly-T tail. To this new product, 10 µl of the modified PCR product was added to 6.5 µl RNase/DNase free water and incubated at 95°C for 2 minutes for opening the double-stranded DNA, the product was then transferred in ice for 2 minutes. Then, 8.5µl of a mixture containing 2.5µl of 10X Tailing buffer, 0.8 mM dTTP, (Genisphere, USA) and 2µl of the enzyme terminal deoxynucleotidyl transferase (Genisphere, USA) were added. The reaction mixture was incubated at 37°C for 30 minutes and subjected immediately to a connection process.

4.20. Ligation of 3 DNA capture sequence

The DNA with the poly-T tail (25µl) was heated at 95°C for 10 minutes and immediately transferred in ice for 2 minutes. Subsequently the mixture was added to 5µl link (Cy3) and 2U of T4 DNA ligase in each sample, which was then incubated at 25°C for 30 minutes. In order to stop the reaction, EDTA was added to obtain a final concentration of 300 mM. The mixture was then homogenized and the volume was adjusted with 1X TE buffer to a final volume of 50µl.

4.21. Purification of Tagged DNA

The tagged DNA was purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer's recommendations. The PCR product was eluted with 15µl of RNase/DNase free water.

4.22. DNA Hybridization

The hybridization buffers (Genisphere, USA) were thawed, heated at 65°C for 10 minutes, homogenized by vortexing and subjected to a brief centrifugation before use. A mix containing 10µl of nuclease free water (Genisphere, USA) and 20µl of 2X hybridization buffer (Genisphere, USA) were added to 10µl of the PCR product. This mixture was heated at 80°C for 10 minutes and then added onto the gasket (Agilent, USA) and subsequently the microarray slide (RoboArboVirusChip) was mounted on the gasket hybridization chamber and incubated overnight at 65°C in a hybridization oven with a rotation of 15 rpm.

4.23. First Wash Post cDNA Hybridization

After hybridization, slides were washed three times to remove non-hybridized PCR products. The wash solution (2X SSC, 0.2% SDS,) was first heated to 65°C. The gasket and the RoboArboChip were removed and washed for 15 minutes in a pre-heated buffer (2X SSC, 0.2% SDS). Subsequently, the slide was washed for 15 minutes at room temperature in 2X SSC and finally for 15 minutes with 0.2 X SSC. The slide was then transferred to a 50 ml tube and centrifuged at 1000 rpm for 2 minutes for drying.

4.24. Hybridization of 3DNA dendrimers

For this second hybridization, 2,5µl of capture reagent of 3DNA dendrimer were mixed with 20µl of 2X hybridization buffer (Genisphere, USA) and 17.5µl RNase/DNase free water to a final volume of 40µl. The mixture was incubated at 80°C for 10 minutes and then applied to the array. The RoboArboChip was again mounted on the hybridization chamber and incubated at 65°C in a hybridization oven with a rotation of 15 rpm for 4 hours. This was followed by washing the slide in the dark as mentioned above.

4.25. Slide scan and data analysis

The slide was scanned at 532 nm using the Axon GenePix 4000B scanner (Molecular Devices, USA) with 10 μ pixel resolution. The obtained image was analyzed with software GenePix Pro 7 to obtain data of fluorescence intensity of each point. Briefly, the GenePix Array List “gal file”, containing the list of probes, was open generating the feature grid, which was positioned on the microarray image and then the fluorescence intensity of spots were obtained. The obtained data were analyzed with the program DetectiV (Watson *et al.*, 2007). Two kinds of files were used, one containing the probes ID and intensities and the other containing the probes annotation file, which has information about species, genus and family of each probes. Probe intensity values were log2 transformed. The mean intensity of the negative control was calculated and used to divide the intensity of the probes for normalization. In case a virus is not present in the sample, it is expected the probes value for this virus to be equal to zero. To analyze if the probes intensity was significantly different from zero, a t-test was carried out. Results were first filtered such that groups had a normalized log2 ratio greater than or equal to 1 (a ratio of two to the control) and then sorted by *p*-value.

3.7.2. Estimated the sensitivity of RoboArboVirusChip and comparison with control slide and qReal-time PCR

The sensitivity of RoboArboVirusChip was compare with control slide. A control slide was designed using sequences available on the slide GreeneChip database Gene Expression Omnibus (GEO) database. The platform selected was GPL10319, which have 45,000 probes. However, the virus species corresponding to each probe is not indicated. For the identification of viral species, each probe was checked by BLAST. To prepare this slide in the same format of eight arrays with 15,000 probes / array, probes corresponding to various controls were removed. Remaining mostly, those corresponding to the virus. In this experiment, we were selected DENV-4 titrated stocks. AxyPrep viral RNA/DNA Miniprep Kit (Axigen, USA) purified total RNA. The RNA were diluted in 10 folds serial dilutions and dilutions 10⁻³ to 10⁻⁸ (4.2x10⁸ to 4.2x10¹ PFU/mL) were used for both slides. All steps same as mention above follow for microarray experiment.

4.26. Detection of mixture of viruses

We used viral RNA of twelve viruses divided in to three pools to evaluate the ability of the RoboArboVirusChip to detect mixture of viruses in a single sample. First pool was mix of viruses represented from three families; BSQV and SLEV (*Flaviviridae*), PIRYV (*Rhabdoviridae*) and MAYV (*Togaviridae*). Second pool was purely belong to flaviviruses: ROCV, SLEV, YFV and DENV-2 and third pool included was the four DENV serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. All viral RNAs derived from cell culture were purified separately and 2.5 µl from each virus were mixed and used for cDNA synthesis. The PCR amplification and microarray experiment were performed as mention above.

4.27. Clinical serum samples

Dengue positive (n=4) and negaive (n=40) serum samples from dengue suspected cases confirmed by RT-PCR were included in the study. All these samples were collected during 2006 – 2012 from deferent primary health care centers at Ribierao Preto and stored in the serum repository of the Virologoy Laboratory at the FCFRP-USP. This study was approved by the Ethical Committee of the FCFRP-USP (CEP/FCFRP nº 313).

5. RESULTS

5.1. Preparation of viral stocks

A total 15 viruses were propagated in C6/36 mosquito cell line for the preparation of viral stocks, which were used for the validation, Lower limits of detection and pools of the RoboArboVirusChip.

5.2. Confirmation of viral infection by indirect immunofluorescence

The infected mosquito C6/36 cells with all viruses were confirmed by indirect immunofluorescence and RT-PCR as illustrated in Figures 2 and 3, respectively.

Figure 2. Indirect immunofluorescence demonstrating infection in C6/36 cells with DENV-2 (B), (C) SLEV, (D) YFV, (E) OROV, and (F) PIRY. (A) Negative control.

Figure 3. Electrophoresis on 1.8% agarose gel showing the DNA bands with the molecular-weight marker as well as negative, positive controls and viruses lane1-Marker, lane-2 DENV-2 (band size 316bp), lane-3 DENV-3 (659bp), lane 4 DENV-4(222bp), lane-5 Busuquara (388bp), lane-6 YFV(253bp), lane -7 Ilheus (474bp). Lane-8 Rocio (230bp), lane -9 SLEV (232bp), lane-10 Negative control-1(during RNA extraction), lane-11 Negative control and lane- 2 Positive control (Rocio virus).

5.3. Determination of viral titer by plaque assay

Viral titer of stocks supernatant was determined by the plaque assay and this supernatant was used for the lower limit of detection of the RoboAboVirusChip (Table 24).

Table 24. Reference virus titers.

Family	Genus	Virus	CPE/ Days	Number of plaques	Viral titer in PFU/ml
<i>Bunyaviridae</i>	Orthobunyavirus	Oropouche virus	8	5/2=2.5	6.25x10 ⁷
		Guaroa virus	7	13/2=6.5	1.6x 10 ⁷
	Hantavirus	Rio Mamore virus			3.0x10 ⁵
<i>Flaviviridae</i>	Flavivirus	Dengue virus type 1	5	4/2=2	5.0x10 ⁴
		Dengue virus type 2	10	2/2=1	2.5x10 ⁸
		Dengue virus type 3	10	4/2=2	5.x10 ⁶
		Dengue virus type 4	10	27	6.75x10 ¹¹
		Yellow fever virus	7	20/2=10	2.5x10 ¹¹
		Bussuquara virus	8	16/2=8	2.0x10 ⁴
		Iguape virus			3.4x10 ⁶
		Ilheus virus	6	18/2=9	2.5x10 ¹¹
		Rocio virus	7	3	7.5x10 ¹⁰
		St.Louis encephalitis virus	7	8/2=4	1.0x10 ⁹
<i>Rhabdoviridae</i>	Vesiculovirus	Piry virus	7	15/2=7.5	1.875x10 ⁴
<i>Togaviridae</i>	Alphavirus	Mayaro virus	6	1	2.5x10 ⁵

5.4. Design of probes for RoboArboVirusChip V1 (version 1) and control slides

In first version of RoboArboVirusChip V1 we selected viruses from five families and eight genera: *Arenaviridae*, *Arenavirus* genus; *Bunyaviridae*, *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* and *Hantavirus* genera; *Flaviviridae*, *Flavivirus* genus, *Rhabdoviridae*, *Vesiculovirus* genus and *Togaviridae*, *Alphavirus* genus. A total 1718 specific viral oligonucleotide probes targeting 239 viruses were designed. In addition, we designed a control slide based on the GreeneChipVr probes list deposited in the GEO

data base (GPL10319). Both RoboArboVirusChip and GreeneChipVr were prepared in a format of eight arrays per slide.

5.5. Implementation of microarray method: Control slide

In the first microarray experiment, we evaluated the performance of the control slide with cDNA and PCR products of four viruses: DENV-2, DENV-3, YFV and BUSV. The cDNA and PCR products were hybridized against the probes contained in the slide arrays. The PCR known products were obtained with primers that amplify a specific a genomic region. The slide was scanned to obtain fluorescence intensity image (Figure 4).

Figure 4. Microarray control slide with 8 arrays scanned at 532 nm. 1. DENV-3 (PCR); 2. DENV-3 (PCR); 3. DENV-2 (cDNA); 4. DENV-3 (cDNA); 5. YFV (cDNA); 6. BUSV (cDNA); DENV-2 (cDNA); and 7. YFV (cDNA).

Figure 5 shows the presence of fluorescence in several spots within the arrays, but also the presence of artifact, which might be due to problem during the washing steps. The feature data from the scanned image was extracted and analyzed with the program GenePix Pro7. Spots with artifacts were excluded from the analysis. The visualization of fluorescence intensity and significant analysis was carried out with the DetectiV program. Figures 6-13 show the visualization of spots fluorescence intensity grouped by family, genus and species levels. While Table 22-29 show the significant analysis of probes for each virus target sorted by *p*-values.

Figure 5. Visualization of probes fluorescence intensity of control slide hybridized with DENV-3 (genomic region: 1-950 bp). Data were sorted by family, genus and species levels.

Table 25. Significant analysis of array hybridized with DENV-3. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	p-value	Average
Lambdapapillomavirus 1	5	0,0148112	2,29
Foot-and-mouth disease virus	6	0,017701354	2,22
Striped bass reovirus	7	0,02206254	1,65
Avian carcinoma virus	5	0,028607965	1,45
Carey Island virus	3	0,030997797	2,07
Cebus albifrons lymphocryptovirus 1	4	0,035129362	2,08
Atractoscion nobilis nervous necrosis virus	5	0,039452058	1,51
Porcine rubulavirus	5	0,039655445	1,18
Murine rotavirus EMcN	3	0,040393312	1,86
Vaccinia virus Tian Tan	3	0,042275962	2,56

FAMILY

Control_13_2.8.1

GENUS

Control_13_2.8.1

Figure 6. Visualization of probes fluorescence intensity of control slide hybridized with DENV-3 (genomic region 8800-10707 bp). Data were sorted by family, genus and species levels.

Table 26. Significant analysis of array hybridized with DENV-3. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -values	average
Dengue virus 3	26	9,49E-10	5,92
Foot-and-mouth disease virus	6	0,00483233	2,29
Carey Island virus	3	0,018282742	2,57
Lambdapapillomavirus 1	5	0,031740476	1,78
Human parechovirus 1	8	0,033104426	1,21
Moloney murine leukemia virus	11	0,034032917	1,12
Striped bass reovirus	7	0,036758429	1,42
Cebus albifrons lymphocryptovirus 1	4	0,039069131	1,68
Atractoscion nobilis nervous necrosis virus	5	0,03914734	1,07
Avian carcinoma virus	5	0,042591587	1,63

FAMILY

Control_13_3.8.1

GENUS

Control_13_3.8.1

Figure 7. Visualization of probes fluorescence intensity of control slide hybridized with DENV-2 (5 µL cDNA). Data were sorted by family, genus and species levels.

Table 27. Significant analysis of array hybridized with DENV-2. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -values	average
Dengue virus 2	40	1,22E-07	2,20
Sepik virus	4	0,002754051	1,13
Dicentrarchus labrax encephalitis virus	15	0,00617462	1,53
Dakar bat virus	4	0,006267702	1,82
Foot-and-mouth disease virus - type SAT 2 (FMDV-SAT2)	6	0,011241274	2,32
Carey Island virus	3	0,01622768	2,70
Lambdapapillomavirus 1	5	0,019340717	2,09
Atractoscion nobilis nervous necrosis virus	5	0,022621486	1,49
Avian carcinoma virus	5	0,027913564	2,21
Moloney murine leukemia virus	11	0,02883574	1,30

FAMILY

Control_13_4.8.1

GENUS

Control_13_4.8.1

Figure 8. Visualization of probes fluorescence intensity of control slide hybridized with DENV-2 (10 μ L cDNA). Data were sorted by family, genus and species levels.

Table 28. Significant analysis of array hybridized with DENV-2. The top 10 viruses with highest p -values.

Virus	No. of probes	p -vavlues	Average
Dengue virus 2	40	1,18E-07	2,14
Dakar bat virus	4	0,002389779	1,65
Sepik virus	4	0,004997352	1,03
Foot-and-mouth disease virus	6	0,010961059	2,24
Dicentrarchus labrax encephalitis virus	15	0,01362258	1,20
Atractoscion nobilis nervous necrosis virus	5	0,014797867	1,05
Carey Island virus	3	0,019887576	2,33
Lambdapapillomavirus 1	5	0,030582442	1,39
Cebus albifrons lymphocryptovirus 1	4	0,031386664	2,15
Avian carcinoma virus	5	0,038736648	1,54

Figure 9. Visualization of probes fluorescence intensity of control slide hybridized with BUSQV. Data were sorted by family, genus and species levels.

Table 29. Significant analysis of array hybridized with BUSQV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Foot-and-mouth disease virus	6	0,002642923	2,17
Carey Island virus	3	0,008005324	2,55
Dicentrarchus labrax encephalitis virus	15	0,012449416	1,30
Aroa virus	10	0,01648986	1,50
Cebus albifrons lymphocryptovirus 1	4	0,029382763	2,14
Striped bass reovirus	7	0,032645494	1,55
Lambdapapillomavirus 1	5	0,049746858	1,56
Macacine herpesvirus 1 (B virus)	3	0,056500526	1,49
Human papillomavirus type 55	3	0,057039384	1,56
Avian carcinoma virus	5	0,057581968	2,13

FAMILY

Control_13_6.8.1

GENUS
Control_13_6.8.1

Figure 10. Visualization of probes fluorescence intensity of control slide hybridized with YFV (5 μ L cDNA). Data were sorted by family, genus and species levels.

Table 30. Significant analysis of array hybridized with YFV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Foot-and-mouth disease virus -	6	0,003351185	2,01
Carey Island virus	3	0,00539798	2,23
Striped bass reovirus	7	0,030127191	1,20
Avian hepatitis E virus	14	0,047470809	1,10
Lambdapapillomavirus 1	5	0,050015069	1,31
Cebus albifrons lymphocryptovirus 1	4	0,051171923	1,34
Avian carcinoma virus	5	0,058107026	1,32
Human papillomavirus type 91 (candHPV91)	7	0,062493027	1,17
Rous-associated virus type 1	3	0,085542893	1,67
Tensaw virus	3	0,088415069	1,87

FAMILY
Control_13_7.8.1

GENUS

Control_13_7.8.1

Figure 11. Visualization of probes fluorescence intensity of control slide hybridized with DENV-2 (5 μ L cDNA). Data were sorted by family, genus and species levels.

Table 31. Significant analysis of array hybridized with DENV-2 . The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Carey Island virus	3	0,001506437	2,66
Foot-and-mouth disease virus - type SAT 2 (FMDV-SAT2)	6	0,003821819	2,25
Dicentrarchus labrax encephalitis virus	15	0,015104683	1,13
Atractoscion nobilis nervous necrosis virus	5	0,029818769	1,06
Avian carcinoma virus	5	0,030631464	1,95
Cebus albifrons lymphocryptovirus 1	4	0,030682021	1,80
Striped bass reovirus	7	0,034686301	1,46
Lambdapapillomavirus 1	5	0,038762179	1,68
Moloney murine leukemia virus	11	0,040688916	1,03
Human parechovirus 1	8	0,061227608	1,06

FAMILY

Control_13_8.8.1

GENUS

Control_13_8.8.1

Figure 12. Visualization of probes fluorescence intensity of control slide hybridized with YFV (5 µL cDNA). Data were sorted by family, genus and species levels.

Table 32. Significant analysis of array hybridized with YFV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Foot-and-mouth disease virus	6	0,008474099	1,68
Carey Island virus	3	0,015505887	2,12
Lambdapapillomavirus 1	5	0,02127612	1,25
Foot-and-mouth disease virus O/SKR/2000	3	0,02862171	1,63
Cebus albifrons lymphocryptovirus 1	4	0,035972593	1,61
Striped bass reovirus	7	0,039311243	1,13
Nodamura virus	7	0,061602055	1,10
Human papillomavirus type 91 (candHPV91)	7	0,062985925	1,09
Avian carcinoma virus	5	0,067795501	1,10
Human parechovirus	5	0,083180856	1,59

The control slide was able to detect DENV-2 and DENV-3, but not YFV neither BUSV. In addition, several cross-hybridization were observed. We did not found any difference between cDNA or PCR products.

5.6. Microarray experiments with control and RoboArboVirusChip v1 slides

A second experiment was performed with the control and RoboArboVirusChip slides, including agitation during the washing steps to reduce the production of artifacts. The following viruses were analyzed with both control and RoboArboVirusChip slides: DENV-2, DENV-3, YFV, BUSV, DENV-4, ILHEV, SLEV and OROV. For the two slides, only cDNA was used. Figures 14 and 15 show the scanned images of control and RoboArboVirusChip slides, respectively, without any artifacts.

Figure 13. Microarray control slide with 8 arrays scanned at 532 nm. DENV-2, DENV-3, YFV, BUSV, DENV-4, ILHEV, SLEV and OROV.

Figure 14. Microarray RoboArboVirusChip slide with 8 arrays scanned at 532 nm. 1DENV-2, DENV-3, YFV, BUSV, DENV-4, ILHEV, SLEV and OROV.

Figure 15. Visualization of probes fluorescence intensity of control slide hybridized with OROV. Data were sorted by family, genus and species levels.

Table 33. Significant analysis of array hybridized with OROV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Foot-and-mouth disease virus	6	0,008474099	1,68
Carey Island virus	3	0,015505887	2,12
Lambdapapillomavirus 1	5	0,02127612	1,25
Foot-and-mouth disease virus O/SKR/2000	3	0,02862171	1,63
Cebus albifrons lymphocryptovirus 1	4	0,035972593	1,61
Striped bass reovirus	7	0,039311243	1,13
Nodamura virus	7	0,061602055	1,10
Human papillomavirus type 91 (candHPV91)	7	0,062985925	1,09
Avian carcinoma virus	5	0,067795501	1,10
Human parechovirus	5	0,083180856	1,59

Figure 16. Visualization of probes fluorescence intensity of control slide hybridized with BUSQV. Data were sorted by family, genus and species levels.

Table 34. Significant analysis of array hybridized with BUSQV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Ross's goose hepatitis B virus	4	0,088137166	1,13
Simian adenovirus 3	5	0,106228303	1,05
Enterovirus T75	4	0,154424677	1,19
Budgerigar fledgling disease polyomavirus (BFPyV)	5	0,176025292	1,10
Human parvovirus B19	2	NA	1,42
Duck circovirus	2	NA	1,30
Uganda S virus	1	NA	2,25
Potiskum virus	1	NA	1,33
Entebbe bat virus	2	NA	1,12
Human papillomavirus type 62	2	NA	2,26

Figure 17. Visualization of probes fluorescence intensity of control slide hybridized with SLEV. Data were sorted by family, genus and species levels.

Table 35. Significant analysis of array hybridized with SLEV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
St. Louis encephalitis virus	176	4,87E-79	4,20
Sepik virus	4	0,000677613	3,41
Bouboui virus	6	0,014903764	1,04
Iguape virus	8	0,037594137	1,11
Aquareovirus A	4	0,083036614	1,27
Vaccinia virus Tian Tan	3	0,089755356	1,01
Ilheus virus	7	0,097969637	1,33
Yaounde virus	3	0,103427328	1,79
Carey Island virus	3	0,107897284	1,66
Ross's goose hepatitis B virus	4	0,109009823	1,06

Figure 18. Visualization of probes fluorescence intensity of control slide hybridized with YFV. Data were sorted by family, genus and species levels.

Table 36. Significant analysis of array hybridized with YFV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Yellow fever virus	70	1,57E-11	1,97
Rocio virus	10	0,004416565	2,08
Ross's goose hepatitis B virus	4	0,088646201	1,35
Aquareovirus A	4	0,120164631	1,09
Budgerigar fledgling disease polyomavirus (BFPyV)	5	0,205378295	1,05
TGEV Miller M60 (TGEV)	3	0,214080666	1,07
Human papillomavirus type 77	3	0,216642785	1,92
Jutiapa virus	3	0,232050769	1,11
Human parvovirus B19	2	NA	2,47
Amur virus	2	NA	1,90

Figure 19. Visualization of probes fluorescence intensity of control slide hybridized with ILHV. Data were sorted by family, genus and species levels.

Table 37. Significant analysis of array hybridized with ILHV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Ilheus virus	7	8,36E-07	5,62
Sepik virus	4	0,000504498	3,70
Aquareovirus A	4	0,080335801	1,12
Human papillomavirus type JEB2	4	0,088757631	1,03
Dakar bat virus	4	0,095624451	2,47
Infectious salmon anemia virus	3	0,163918924	1,13
Kadam virus	3	0,199990503	1,42
Budgerigar fledgling disease polyomavirus (BFPyV)	5	0,208990471	1,02
Human parvovirus B19	2	NA	2,06
Duck circovirus	2	NA	1,12

Figure 20. Visualization of probes fluorescence intensity of control slide hybridized with DENV-3. Data were sorted by family, genus and species levels.

Table 38. Significant analysis of array hybridized with DENV-3. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Dengue virus 3	26	3,49E-09	4,10
Ross's goose hepatitis B virus	4	0,091616271	1,18
Israel turkey meningoencephalomyelitis virus	6	0,092116512	1,92
Aquareovirus A	4	0,093947029	1,08
Infectious salmon anemia virus	3	0,176806457	1,11
Human parvovirus B19	2	NA	1,87
Duck circovirus	2	NA	1,18
Potiskum virus	1	NA	1,33
Human papillomavirus type 62	2	NA	2,32
Human papillomavirus type 15	2	NA	1,14

Figure 21. Visualization of probes fluorescence intensity of control slide hybridized with DENV-4. Data were sorted by family, genus and species levels.

Table 39. Significant analysis of array hybridized with DENV-4. The top 10 viruses with lowest p -values.

Virus	No. opf probes	<i>p</i> -value	Average
Dengue virus 4	43	3,32E-09	3,56
Bouboui virus	6	2,85E-06	5,87
Aroa virus	10	0,000994282	3,56
Sepik virus	4	0,024625891	2,96
Iguape virus	8	0,045791538	1,53
Aquareovirus A	4	0,047480907	1,28
Kyasanur forest disease virus	5	0,10878446	1,01
Stratford virus	6	0,121011845	1,90
Ross's goose hepatitis B virus	4	0,123320852	1,26
Sagiyama virus	3	0,124897547	1,30

Control_8.8.1

Figure 22. Visualization of probes fluorescence intensity of control slide hybridized with DENV-2. Data were sorted by family, genus and species levels.

Table 40. Significant analysis of array hybridized with DENV-2. The top 10 viruses with lowest p -values.

Virus	No. of probes	p-value	Average
Dengue virus 2	40	3,27E-09	2,99
Sepik virus	4	0,001131728	2,19
Simian cytomegalovirus	13	0,00185129	1,51
Avian carcinoma virus	5	0,002049661	1,90
Sapporo virus	17	0,002101187	1,12
Chipmunk parvovirus (ChPV)	10	0,00524159	1,66
Dakar bat virus	4	0,007803725	3,49
Cercopithecine herpesvirus 2 (Simian agent 8)	10	0,008182422	1,36
Atractoscion nobilis nervous necrosis virus	5	0,008779628	1,91
Dicentrarchus labrax encephalitis virus	15	0,00943468	1,21

The control slide was able to detect SLEV, YFV, ILHV, DENV-4, DENV-3, and DENV-2, but not OROV neither BUSV. After analysis of the probes included in the control slide, we found that no probes were included for BUSQV. In addition, several probes with cross-hybridization were found in both experiments using this slide. We concluded that this slide is not matching the standard as a control slide; that is why we decided not to use farther in this study.

Figure 23. Visualization of probes fluorescence intensity of control slide hybridized with OROV. Data were sorted by family, genus and species levels.

Table 41. Significant analysis of array hybridized with OROV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Edge Hill virus	11	1,15E-12	1,40
Muleshoe virus	11	6,40E-08	1,58
Cy1014 virus	22	4,95E-05	2,07

Figure 24. Visualization of probes fluorescence intensity of control slide hybridized with BUSQV. Data were sorted by family, genus and species levels.

Table 42. Significant analysis of array hybridized with BUSQV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Bussuquara virus	44	6,16E-22	1,84
Edge Hill virus	11	1,16E-11	1,22
Muleshoe virus	11	3,46E-08	1,63
Cy1014 virus	22	5,19E-05	1,98

FAMILY

RoboArbochip_results_3.8.1

GENUS

RoboArbochip_results_3.8.1

Figure 25. Visualization of probes fluorescence intensity of control slide hybridized with SLEV. Data were sorted by family, genus and species levels.

Table 43. Significant analysis of array hybridized with SLEV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Stratford virus	11	1,53E-16	4,06
Edge Hill virus	11	1,36E-11	1,45
Muleshoe virus	11	9,95E-09	1,92
St. Louis encephalitis virus	11	6,74E-08	1,65
Cy1014 virus	22	3,91E-05	2,27

Figure 26. Visualization of probes fluorescence intensity of control slide hybridized with YFV. Data were sorted by family, genus and species levels.

Table 44. Significant analysis of array hybridized with YFV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Yellow fever virus	33	3,68E-37	3,57
Edge Hill virus	11	4,32E-11	1,27
Muleshoe virus	11	6,44E-08	1,57
Cy1014 virus	22	5,62E-05	1,94

Figure 27. Visualization of probes fluorescence intensity of control slide hybridized with ILHV. Data were sorted by family, genus and species levels.

Table 45. Significant analysis of array hybridized with ILHV. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Ilheus virus	22	5,85E-37	4,446324797
Edge Hill virus	11	5,34E-12	1,773708468
Guanarito virus	55	7,07E-09	1,079062403
Bujaru virus	11	1,26E-08	1,362636889
Muleshoe virus	11	7,39E-08	2,476204129
Cy1014 virus	22	5,16E-05	2,317890492

FAMILY

RoboArbochip_results_6.8.1

GENUS

RoboArbochip_results_6.8.1

Figure 28. Visualization of probes fluorescence intensity of control slide hybridized with DENV-3. Data were sorted by family, genus and species levels.

Table 46. Significant analysis of array hybridized with DENV-3. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Dengue virus type 3	44	4,35E-10	2,01
Edge Hill virus	11	7,43E-10	1,22
Muleshoe virus	11	1,14E-08	1,72
Cy1014 virus	22	8,59E-05	1,88

FAMILY
RoboArbochip_results_7.8.1

GENUS
RoboArbochip_results_7.8.1

Figure 29. Visualization of probes fluorescence intensity of control slide hybridized with DENV-4. Data were sorted by family, genus and species levels.

Table 47. Significant analysis of array hybridized with DENV-4. The top 10 viruses with lowest p -values.

Virus	No. of probes	p -value	Average
Dengue virus type 4	110	1,60E-54	3,35
Edge Hill virus	11	1,03E-10	1,24
Muleshoe virus	11	4,06E-10	2,71
Cy1014 virus	22	4,32E-05	2,00

FAMILY

RoboArbochip_results_8.8.1

GENUS

RoboArbochip_results_8.8.1

Figure 30. Visualization of probes fluorescence intensity of control slide hybridized with DENV-2. Data were sorted by family, genus and species levels.

Table 48. Significant analysis of array hybridized with DENV-2. The top 10 viruses with highest p -values.

Virus	No. of probes	p -value	Average
Dengue virus type 2	44	4,33E-53	3,866424998
Edge Hill virus	11	3,67E-10	1,296571693
Muleshoe virus	11	8,79E-07	1,53532919
Cy1014 virus	22	7,36E-05	2,003253838

The RoboArboVirusChip v1 slide was able to detect SLEV, YFV, ILHV, DENV-4, DENV-3, DENV-2, and BUSV but not OROV. However, cross-hybridization with the following viruses were also observed: Edge Hill virus, Muleshoe virus, Cy1014 virus, Edge Hill virus, Guanarito virus, Bujaru virus, Muleshoe virus, Cy1014 virus and Stratford virus. We have analyzed which probes were those showing cross-hybridization and removed and replaced with new probes for the next version. In addition, we included new probes for OROV.

5.7. RoboArboVirusChip second version (V2)

To prepare RoboArboVirusChip V2, first we removed probes belonging to viruses mentioned above. In addition, we have included probes for other 16 viruses within the families mentioned in the first version and viruses of a new family *Reoviridae*, *Orbivirus* genus. This version contained total of 1743 specific oligonucleotide probes for targeting 255 viruses. Selection of probes and preparation of microarray slide was the same as RoboArboVirusChip v1. In the experiments with this slide, we started to use a positive RNA control (spike) provided by Agilent.

To analyze this new slide, we have used a cDNA of eight viruses: DENV-1, DENV-2, YFV, PIRYV, BUSQV, OROV, DENV-3 and DENV-4 (Tables 41-48). The hybridization experiments and data analysis were performed as mentioned above. Tables 25-32 showed the significant analysis of data of each array.

Table 49. Significant analysis of array hybridized with DENV-1. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Dengue virus type 1	44	6,08E-18	5,04
Spike	20	1,49E-14	1,09
Turuna virus	81	1,37E-10	1,70
Edge Hill virus	9	6,22E-08	3,00
Muleshoe virus	9	2,34E-06	4,00
Guanarito virus	54	6,92E-06	1,37
Frijoles virus	36	4,22E-05	1,63

Table 50. Significant analysis of array hybridized with DENV-2. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	1,78E-31	2,40
Dengue virus type 2	36	4,99E-10	2,03

Table 51. Significant analysis of array hybridized with YFV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	6,42E-36	2,76
Yellow fever virus	63	4,57E-08	1,00

Table 52. Significant analysis of array hybridized with DENV-1. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i>-value	Average
Controle positivo 135	20	7,73E-16	5,10
Piry virus	45	7,00E-11	1,08
Equine encephalosis virus	135	2,86E-10	1,14
Candiru virus	99	3,31E-10	1,42
Spike	20	5,26E-10	1,32
Turuna virus	81	3,26E-09	1,79
Muleshoe virus	9	2,86E-08	5,04
Frijoles virus	36	3,45E-08	2,64
Edge Hill virus	9	1,94E-06	4,00

Table 53. Significant analysis of array hybridized with BUSQV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	3,39E-28	1,85
Bussuquara virus	36	1,70E-21	5,23
Muleshoe virus	9	4,31E-05	2,87
Frijoles virus	36	0,000126872	1,20

Table 54. Significant analysis of array hybridized with OROV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i>-value	Average
Oropouche virus	126	3,18E-56	4,55

Spike	200	4,92E-31	2,19
Muleshoe virus	9	4,02E-08	3,77
Frijoles virus	36	0,00010446	1,50

Table 55. Significant analysis of array hybridized with DENV-3. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Muleshoe virus	9	5,43E-07	1,57
Dengue virus type 3	36	3,06E-05	2,14
Spike	200	2,11E-20	1,09

Table 56. Significant analysis of array hybridized with DENV-4. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Dengue virus type 4	90	3,58E-43	4,76
Muleshoe virus	9	1,34E-09	1,46
Spike	200	8,14E-36	2,75
Broadhaven virus	45	2,11E-10	2,08
Agilent_Bright corner control	14	6,17E-09	2,64
Agilent_Internal control	36	3,57E-08	2,51
Muleshoe virus	9	1,39E-07	1,92
Frijoles virus	36	8,46E-05	1,07
Edge Hill virus	9	0,000163025	1,56

The results showed that all analyzed viruses were specifically detected; although cross-hybridization was also observed in this version.

5.8. Comparison of cDNA and PCR products as target for the RoboArboVirusChip v2

The virus load found in clinical samples, especially in serum samples are lower than the viral load found in the supernatant of cell cultures. Therefore, the inclusion of virus genomic amplification by PCR would improve the sensitivity of the RoboArboVirusChip v2 when using serum samples. To test this hypothesis, we have compared the sensitivity of RoboArboVirusChip v2 to detect cDNA and one step and two steps RT-PCR products of YFV (2.5×10^{11} PFU/mL) and DENV-2 (2.5×10^8 PFU/mL). In this experiment we have used cDNA and a one-step RT-PCR products obtained from virus RNAs diluted 1:10 and 1:1000 (Table 49). The RT-PCR was performed using random primers as described in Material and Methods.

Table 57. Significant analysis of RoboArboVirusChip v2 hybridized with cDNA and PCR products of YFV. The top 10 viruses with lowest *p*-values.

Targuet	Titer (PFU/mL)	Virus	No. of probes	<i>p</i> -value	Average
cDNA	2.5×10^{10}	Spike	200	1,68E-13	2,95
	2.5×10^8	Spike	200	5,93E-11	2,51
One-step RT-PCR	2.5×10^{10}	Spike	200	2,50E-17	3,93
		Yellow fever virus	63	2,38E-12	2,55
	2.5×10^8	Spike	200	2,67E-14	2,73
		Yellow fever virus	63	4,57E-08	1,00
	2.5×10^7	Spike	200	2,97E-20	4,11
Two step RT-PCR	2.5×10^{10}	Spike	200	7,37E-14	2,05
		Yellow fever virus	63	4,31E-40	6,70
	2.5×10^8	Spike	200	7,17E-49	3,35
		Yellow fever virus	63	1,05E-21	3,45

2.5x10 ⁷	Spike	200	3,41E-15	5,86
	Yellow fever virus	63	2,43E-11	1,55

The RoboArboVirusChip v2 was able to detect the viruses using PCR products, and the two steps RT-PCR was the better one. Therefore, we decided to use the two steps RT-PCR in the farther next all experiments.

5.9. Detection of mixture of viruses by the RoboArboVirusChip v2

Infection with more than one virus is a likely event that can be seen in human, especially for arboviruses. In this experiment, we have evaluated the ability of RoboArboVirusChip to detect mixture of viruses in a single sample, we used two pools of viral RNA of eight flaviviruses, each pool contained four viral RNAs: 1) ROCV, SLEV, YFV and DENV-2; and 2) DENV-1, DENV-2, DENV-3 and DENV-4. The microarray experiment and data analysis were performed as mention above (Table 55).

Table 58. Significant analysis of arrays hybridized with pool of viruses. The top 10 viruses with lowest *p*-values.

Pools	Virus	No. of probes	<i>p</i> -value	Average
1	Dengue virus type 4	90	9,46E-44	5,28
	Dengue virus type 2	36	7,48E-29	4,47
	Dengue virus type 1	45	1,42E-16	3,26
	Turuna virus	81	1,24E-08	1,20
	Edge Hill virus	9	3,60E-08	1,86
	Muleshoe virus	9	2,83E-06	3,44
	Dengue virus type 3	36	2,84E-06	2,78
	Guanarito virus	54	8,22E-06	1,08
	Frijoles virus	36	2,03E-05	1,40
	Snowshoe Hare virus	18	0,000641864	1,21
2	Rocio virus	72	2,64E-72	6,25

Dengue virus type 2	36	5,53E-34	5,29
Yellow fever virus	63	1,56E-27	5,28
Spike	200	2,14E-27	1,73
St. Louis encephalitis virus	72	2,85E-26	4,58
Aura virus	27	7,68E-18	3,38
Arbia virus	36	7,09E-11	2,66
Turuna virus	81	6,18E-08	1,05
Edge Hill virus	9	7,74E-07	1,68
Guanarito virus	54	3,13E-06	1,16

The RoboArboVirusChip v2 was able to specifically detect all viruses within the two pools. However, some probes cross-hybridized with non-target viruses.

5.10. Determination of sensitivity of the RoboArboVirusChip v2

The sensitivity of the RoboArboVirusChip was determined by using 10 fold-serial dilutions of DENV-2 (2.5×10^8)/ 2.5×10^9 and DENV-4 (6.75×10^{11})/ 2.1×10^9 . Microarray experiment was performed as mention above (Table 56-57).

Table 59. Determination of sensitivity of the RoboArboChip v2 for DENV-2.

Titer (PFU/mL)	Virus	No. of probes	<i>p</i> -value	Average
2.5×10^6	Spike	200	5,15E-50	3,80
	Dengue virus type 2	36	7,23E-30	5,40
	Turuna virus	81	4,31E-09	1,68
	Muleshoe virus	9	2,24E-08	4,99
	Guanarito virus	54	5,10E-06	1,55
	Frijoles virus	36	5,18E-06	2,20
	Edge Hill virus	9	1,04E-05	3,04
	Snowshoe Hare virus	18	0,000330158	1,87
	Rio Bravo virus	18	0,000407347	1,83

2.5x10 ⁵	Spike	200	2,17E-70	5,27
	Dengue virus type 2	36	9,38E-22	5,02
	Turuna virus	81	2,72E-09	1,55
	Edge Hill virus	9	6,15E-08	2,71
	Muleshoe virus	9	7,04E-07	4,39
	Guanarito virus	54	2,73E-06	1,50
	Frijoles virus	36	7,36E-06	2,03
	Punta Salinas virus	18	0,000218475	1,25
	Snowshoe Hare virus	18	0,000266901	1,86
2.5x10 ⁴	Spike	200	3,65E-39	2,57
	Edge Hill virus	9	3,84E-07	1,43
	Muleshoe virus	9	3,45E-05	2,11
2.5x10 ³	Spike	200	1,88E-42	4,06
	Turuna virus	81	9,45E-09	1,17
	Guanarito virus	54	3,73E-06	1,19
	Edge Hill virus	9	7,18E-06	1,80
	Muleshoe virus	9	2,28E-05	3,28
	Frijoles virus	36	3,05E-05	1,38
	Snowshoe Hare virus	18	0,001678851	1,17
2.5x10 ²	Spike	200	1,07E-45	3,88
	Turuna virus	81	6,06E-08	1,30
	Edge Hill virus	9	8,31E-06	2,27
	Frijoles virus	36	1,32E-05	1,82
	Muleshoe virus	9	1,56E-05	4,17
	Guanarito virus	54	2,24E-05	1,28
	Snowshoe Hare virus	18	0,000787344	1,42
	Thailand virus	36	0,000960894	1,15
	Gadgets Gully virus	18	0,001377639	1,08

25	Spike	200	1,07E-53	4,79
	Turuna virus	81	1,02E-08	1,38
	Edge Hill virus	9	6,05E-08	2,12
	Muleshoe virus	9	5,12E-07	4,27
	Guanarito virus	54	6,69E-06	1,35
	Frijoles virus	36	1,92E-05	1,74
	Rio Bravo virus	18	0,000681044	1,54
	Snowshoe Hare virus	18	0,000837261	1,40
	Oriboca virus	27	0,000987481	1,13

Table 60. Determination of sensitivity of the RoboArboChip for DENV-4.

Dilution	Virus	No. of probes	p-value	Average
2.1x10 ⁶	Dengue virus type 4	90	3,40E-40	5,81
	Spike	200	1,26E-30	8,04
	Muleshoe virus	9	3,96E-10	4,17
	Candiru virus	99	4,90E-10	1,02
2.1x10 ⁵	Dengue virus type 4	90	1,33E-40	6,34
	Spike	200	3,23E-28	8,82
	Turuna virus	81	7,15E-09	1,18
	Muleshoe virus	9	7,80E-09	1,95
2.1x10 ⁴	Dengue virus type 4	90	9,00E-40	5,67
	Spike	200	1,46E-32	8,69
	Turuna virus	81	1,91E-08	1,43
	Muleshoe virus	9	6,47E-08	2,29
2.1x10 ³	Dengue virus type 4	90	8,36E-30	3,25
	Spike	200	9,16E-27	8,30
	Turuna virus	81	9,25E-09	1,19

	Muleshoe virus	9	1,93E-08	3,20
2.1x10 ²	Spike	200	5,25E-17	6,11
	Turuna virus	81	2,59E-08	1,30
	Frijoles virus	36	9,49E-06	1,71
21	Spike	200	6,94E-16	4,63
	Guanarito virus	54	3,51E-05	1,02
	Muleshoe virus	9	5,92E-05	2,35

The RoboArboVirusChip V2 was able to detect as few as 2.5x10⁵ PFU/mL of DENV-2 and 2.1x10³ PFU/mL of DENV-4.

5.11. Design of the RoboArboVirusChip v3

We found some probes from the following viruses with cross hybridization in the RoboArboVirusChip v2: Muleshoe virus, Edge Hill virus, Equine encephalosis virus, Frijoles virus, Broadhaven virus, Guanarito virus, Snowshoe Hare virus, Rio Bravo virus, Oriboca virus, Gadgets Gully virus, Punta Salinas virus, Thailand virus and Turuna virus. All probes showing cross-hybridization were removed and replaced by new ones to design the RoboArboVirusChip v3. In this version, we included the fallowing new families and their genera: *Reoviridae*; Coltivirus and Seadornavirus genera; *Rhabdoviridae*, Ephemerovirus genus; and *Asfarviridae*, *Asfarvirus* genus. In this version, we have also included generic probes to detect viruses at the genus level. These probes were designed from conserved RNA genomic regions of representative viruses within each genus. A total 4296 oligonucleotide probes were selected, including 4209 specific probes to target 412 virus species and 87 generic probes (Supplementary Table 1).

This new slide was tested with 17 viruses (BUSQV, CHIKV, DENV-1, DENV-2, DENV-3, DENV-4, GROV, IGUV, ILHV, MAYV, OROV, PIRYV, RIOMV, ROCV,

SLEV, WNV and YFV) and RNA obtained from uninfected C6/36 cells as a negative control. Visualization of probes intensity sorted by families, genera and species for the array hybridized with DENV-3 are shown in Figure 32; while significance analysis is shown in Table 58.

Table 61. Significance analysis of arrays hybridized with DENV-3. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	1,82E-14	1,02
Dengue virus type 3	39	1,72E-12	4,28
Agilent_Internal control	36	6,09E-05	1,19
NA	NA	NA	NA

In Figures 33-48, we are showing the visualization of probes intensity of arrays hybridized with BUSQV, CHIKV, DENV-1, DENV-2, DENV-4, GROV, IGUV, ILHV, MAYV, OROV, PIRYV, RIOMV, ROCV, SLEV, WNV, YFV and negative control. The significance analysis of data for these arrays are shown in Tables 59-75,

Table 62. Significance analysis of arrays hybridized with BUSQV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	3,01E-23	1,80
Bussuquara virus	36	1,00E-17	5,56
Agilent_Internal control	36	0,000113093	1,56
NA	NA	NA	NA

Figure 33. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with CHIKV. Data were sorted by species of the *Alphavirus* genus levels.

Table 63. Significance analysis of arrays hybridized with CHIKV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	1,22E-06	1,20
Chikungunya virus	34	4,59E-06	2.81
NA	NA	NA	NA

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	7,69E-45	3,71
Dengue virus type 1	36	7,02E-21	6,18
Agilent_Internal control	36	1,17E-07	2,80
NA	NA	NA	NA

Figure 35. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with DENV-2. Data of virus species within the *Flavivirus* genus are shown.

Table 65. Significance analysis of arrays hybridized with DENV-2. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	4,15E-43	3,76
Dengue virus type 2	36	9,71E-16	5,68
Agilent_Internal control	36	5,65E-08	3,06
NA	NA	NA	NA

Table 66. Significance analysis of arrays hybridized with DENV-4. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	4,76E-20	1,48
Dengue virus type 4	31	1,69E-05	2,23
Agilent_Internal control	36	3,46E-05	1,69
NA	NA	NA	NA

Figure 37. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with GROV. Data of virus species within the *Orthobunyavirus* genus are shown.

Table 67. Significance analysis of arrays hybridized with GROV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	3,66E-38	3,24
Guaroa virus	36	1,15E-32	6,69
Agilent_Internal control	36	7,14E-07	2,63
NA	NA	NA	NA

Figure 38. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with IGUV. Data of virus species within the *Flavivirus* genus are shown.

Table 68. Significance analysis of arrays hybridized with IGUV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	4,88E-40	3,67
Iguape virus	39	8,47E-27	6,80
Agilent_Internal control	36	7,29E-08	3,15
NA	NA	NA	NA

Table 69. Significance analysis of arrays hybridized with ILHV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	12,50784687	2,08
Ilheus virus	38	18,59038861	5,42
Agilent_Internal control	36	4,708345624	1,82
Spike	200	12,50784687	2,08

Figure 40. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with MAYV. Data of virus species within the *Alphavirus* genus are shown.

Table 70. Significance analysis of arrays hybridized with MAYV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	2,11E-36	3,28
Mayaro virus	37	1,42E-07	3,24
Agilent_Internal control	36	2,57E-07	2,83
NA	NA	NA	NA

Virus	No. of probes	<i>p</i>-value	Average
Oropouche virus	82	2,64E-34	4,72
Spike	200	2,63E-27	2,31
Agilent_Internal control	36	6,90E-05	1,91
NA	NA	NA	NA

Figure 42. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with PIRYV. Data of virus species within the *Vesiculovirus* genus are shown.

Table 72. Significance analysis of arrays hybridized with PIRYV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	4,04E-31	1,90
Piry virus	40	1,50E-18	5,73
Agilent_Internal control	36	4,08E-05	1,29
NA	NA	NA	NA

Figure 43. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with RIOMV. Data of virus species within the *Hantavirus* genus are shown.

Table 73. Significance analysis of arrays hybridized with RIOMV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	1,22E-59	4,49
Rio Mamore virus	72	7,14E-21	3,35
Agilent_Internal control	36	1,12E-07	2,96
NA	NA	NA	NA

Figure 44. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with ROCV. Data of virus species within the *Flavivirus* genus are shown.

Table 74. Significance analysis of arrays hybridized with ROCV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	3,64E-12	3,61
Rocio virus	33	1,43E-10	4,23
NA	NA	NA	NA

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	2,37E-24	1,71
St. Louis encephalitis virus	34	5,35E-14	5,25
Agilent_Internal control	36	0,000296699	1,24
NA	NA	NA	NA

Table 76. Significance analysis of arrays hybridized with WNV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
West Nile virus	34	12,27891334	4,92
Spike	200	15,21410118	3,74
NA	NA	NA	NA

Table 77. Significance analysis of array hybridized with YFV. The top 10 viruses with lowest p -values.

Virus	No. of probes	<i>p</i>-value	Average
Spike	200	9,80E-20	1,43
Yellow fever virus	36	9,62E-11	4,46
Agilent_Internal control	36	5,17E-05	1,53
NA	NA	NA	NA

Figure 48. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with RNA of uninfected C6/36 cells. Data of virus species within the *Flavivirus* genus are shown.

Table 78. Significance analysis of array hybridized with RNA of uninfected C6/36 cells. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Spike	200	6.53E-37	2.91
Agilent_Internal control	36	1.11E-06	2.42
NA	NA	NA	NA

5.12. Detection of mixture of viruses by the RoboArboVirusChip v3

We tested the ability of this version to detect the mixture of viruses in a single sample. Two pools, one containing RNAs of viruses from three families, *Togaviridae* (MAYV), *Flaviviridae* (SLEV and BUSQV) and *Rhabdoviridae* (PIRYV), and the other containing RNAs of the four DENV serotypes were used. The visualizations of spots intensity are shown in Figures 50-53 and the significance analysis of the array hybridized with viruses from three different families is shown in Tables 76-77.

Figure 49. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with MAYV, SLEV, BUSQV and PIRYV. Data of virus species within the *Flavivirus* genus are shown.

Figure 50. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with MAYV, SLEV, BUSQV and PIRYV. Data of virus species within the *Alphavirus* genus are shown.

Figure 51. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with MAYV, SLEV, BUSQV and PIRYV. Data of virus species within the *Vesiculovirus* genus are shown.

Table 79. Significance analysis of array hybridized with a pool of MAYV, SLEV, BUSQV and PIRYV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
St. Louis encephalitis virus	34	2,55E-15	4,35
Spike	200	1,39E-13	4,44
Piry virus	40	2,04E-13	3,50
Bussuquara virus	36	2,20E-13	2,60
Myaro virus	37	4,54E-12	3,18
NA	NA	NA	NA

The visualizations of spots intensity are shown in Figures 53 and the significance analysis of the array hybridized with the four DENV serotypes is shown in Table 72.

Figure 52. Visualization of probes fluorescence intensity of RoboArboVirusChi v3 slide hybridized with DENV-1, DENV-2, DENV-3 and DENV-4. Data of virus species within the *Flavivirus* genus are shown.

Table 80. Significance analysis of array hybridized with a pool of MAYV, SLEV, BUSQV and PIRYV. The top 10 viruses with lowest *p*-values.

Virus	No. of probes	<i>p</i> -value	Average
Dengue virus type 3	39	8,23E-27	6,03
Spike	200	3,23E-25	6,40
Dengue virus type 2	36	1,01E-22	5,88
Dengue virus type 1	36	2,34E-20	5,26
Dengue virus type 4	31	2,07E-13	4,86

The RoboArboVirusChip v3 was able to specifically detect all the viruses within the two pools.

5.13. Determination of sensitivity of the RoboArboVirusChip v3

The sensitivity of the RoboArboVirusChip v3 was determined by using 10 fold-serial dilutions of DENV-1 (2.6×10^7 RNA copies/mL), DENV-2 (2.5×10^9 RNA copies/mL), DENV-3 (6.7×10^7 RNA copies/mL) and DENV-4 (2.1×10^9 RNA copies/mL). Microarray experiment was performed as mention above (Table 78).

Table 81. Determination of sensitivity of the RoboArboVirusChip v3 for DENV-1, DENV-2, DENV-3 and DENV-4.

Virus	Titer	<i>p</i>-value	Average	Spike <i>p</i>-value	Spike average
(RNA copies/mL)					
DENV-1	26	1.58E-06	1.619	6.77E-27	7.610
DENV-2	25	2.57E-07	2.156	6.10E-29	8.081
DENV-3	67	1.23E-12	4.333	3.76E-30	8.006
DENV-4	21	2.99E-21	2.592	1.85E-54	5.601

5.14. Analysis of clinical samples with the RoboArboVirusChip v3

Serum samples collected from DENV infected (n=4) and uninfected (n=40) patients were used to determine the applicability of the RoboArboVirusChip v3 for detecting virus in serum samples. A pool of four serum samples from uninfected patients were analyzed per array, all of which showed a negative result in the microarray assay (Table 79). While all serum samples of DENV infected patients (DENV-1=1, DENV-2=1, DENV-3, and DENV-4=1), analyzed in independent arrays, were specifically detected (Table 80).

Table 82. Significance analysis of array hybridized with RNA of serum samples of DENV uninfected cases. The top 10 viruses with lowest *p*-values.

Samples ID	Virus	No. of probes	<i>p</i> -value	Average
1, 2, 2664 and 1328	Spike	200	2,11E-20	1,09
	NA	NA	NA	NA
2671, 1318, 2666 and 1390	Spike	200	8,14E-36	2,75
	Agilent_Internal control	36	3,57E-08	2,51
1334, 1337, 1323 and 1239	NA	NA	NA	NA
	Spike	200	1,22E-29	2.06
2082, 2272, 2076 and 2306	NA	NA	NA	NA
	Spike	200	6.27E-31	2.20
2271, 2091, 2051 and 2109	NA	NA	NA	NA
	Spike	200	7.33E-50	3.94
2112, 2238, 2086 and 2265	NA	NA	NA	NA
	Spike	200	1.35E-41	2.43
2262, 2075, 2069 and 2240	NA	NA	NA	NA
	Spike	200	1.33E-17	1.37
2290, 2270, 2289 and 1223	NA	NA	NA	NA
	Spike	200	1.88E-46	3.71
1298, 1325, 1847 and 1651	NA	NA	NA	NA
	Spike	200	2.71E-45	3.61
2488, 2510, 2309 and 2516	NA	NA	NA	NA
	Spike	200	5.46E-27	1.91
	NA	NA	NA	NA

Table 83. Significance analysis of array hybridized with RNA of serum samples of DENV infected cases. The top 10 viruses with lowest p -values.

Samples ID	Virus	No. of probes	p -value	Average
2116	Spike	200	9,87E-60	4,49
	Agilent_Internal control	36	1,14E-06	2,58
	Dengue virus type 3	36	0,003032398	1,04
	NA	NA	NA	NA
1111	Spike	200	2,24E-14	2,12
	Agilent_Internal control	36	7,65E-08	3,07
	Dengue virus type 4	31	4,26E-07	2,51
	NA	NA	NA	NA
1112	Dengue virus type 1	36	1,23424E-05	2,42
	Spike	200	6.27E-31	2.20
	NA	NA	NA	NA
1113	Dengue virus type 1	36	2,43424E-06	1,54
	Spike	200	5.237E-25	2.35
	NA	NA	NA	NA

6. DISCUSSION

The differential diagnosis of infectious disease is a great challenge in clinical medicine, public and veterinary health, especially during epidemic outbreaks. The initial sign and symptoms caused by roboviruses and arboviruses infecting humans are very similar, therefore specific laboratory test are necessary for the final diagnosis. In this study we have developed a microarray platform that can be used in the surveillance of all roboviruses and arboviruses with sequences deposited in the GenBank.

The early identification of viruses during epidemic outbreaks helps the health authorities to implement the more appropriated disease containment strategies. Conventional methods of virus identification include immunoassays, PCRs and virus isolation; however these methods can detect only one or few viruses in a single assay, turning the final diagnosis laborious, time consuming and expensive when several assays are needed (Elnifro *et al.*, 2000). The DNA microarray platform developed in this study can be used to screen up to 412 viruses in a single assay, allowing an early identification of an outbreak causative agent. We have evaluated this platform with 17 reference viruses and all of them were readily detected and identified by specific hybridization to

the appropriate oligonucleotides probes. Unfortunately, the genus specific probes were not able to detect the virus at the genus level, suggesting this platform could be not use for new virus discovery.

The RoboArboVirusChip designed in this study contain up to 10 probes for 412 viruses with sequences deposited in the GenBank up to February 2012. However, new probes designed based on new sequences deposited in the GenBank for those viruses with less than 10 probes will be updated and tested in the future. To our knowledge, this is only DNA microarray platform containing probes aiming the specific identification of roboviruses and arboviruse. Although the DNA microarray platform called GreenechipVr was designed to detect all the vertebrate viruses (1710 viruses, including roboviruses and arboviruses) (Palacios, 2007), this platform covers viruses with sequences deposited in the GenBank up to 2007. For this reason, the RoboArboVirusChip covers an additional of 183 viruses when compared to the GreenechipVr. The viruses covered by the RoboArboVirusChip and not by the GreenechipVr belong to the following families: *Arenaviridae* (19 viruses), *Bunyaviridae* (123 viruses), *Flaviviridae* (7 viruses), *Reoviridae* (16 viruses), *Rhabdoviridae* (15 viruses) and *Togaviridae* (3 viruses). Therefore, our platform has a better capability to detect roboviruses and arboviruse than the GreenechipVr. In addition, GreenechipVr includes less than 10 probes for several roboviruses and arboviruses, suggesting that is less sensitive to detect those viruses when compared to the RoboArboVirusChip, which contain at least 10 probes for most of those viruses.

Although our platform is the only one targeting roboviruses and arboviruses, some studies in the literature describe the development of DNA microarray tools containing probes for arboviruses. For example, there are DNA microarray platforms designed to identify: *Dengue virus* serotypes (Putonti *et al.*, 2006), seven flaviviruses pathogenic for human (Nordström *et al.*, 2005), flaviviruses causing infections of the central nervous system (Korimbocus *et al.*, 2005), and seven European bunyaviruses causing encephalitis or meningo-encephalitis (Hasib *et al.*, 2011). In addition, the ArboChip3.0 was designed to detect 33 flaviviruses pathogenic for human (Grubaugh, Petz, *et al.*, 2013), and then updated to the ArboChip5.1 version to detect 144 RNA viruses transmitted by mosquitoes from four arbovirus genera (*Alphavirus*, *Flavivirus*, *Orthobunyavirus* and *Phlebovirus*) (Grubaugh, Mcmenamy, *et al.*, 2013). All those studies targeted less number of arboviruses when compared to our RoboArboVirusChip.

In addition, our platform target all pathogenic and non-pathogenic arboviruses for human and livestock. In some cases, non-pathogenic viruses easily can turn emergent viruses. For example, Rio Mamore virus was previously considered non-pathogenic for human but recently fatal Hantavirus Pulmonary Syndrome (HPS) cases have been reported in Peru, French Guiana and Brazilian Amazon regions associated with this virus (Casapía *et al.*, 2012; Matheus *et al.*, 2012; De Oliveira *et al.*, 2014).

Other studies in the literature describe the development of DNA microarray tools targeting arboviruses. In one such study, 47 probes targeting only *Lassa virus* were designed (Olschläger and Günther, 2012). Another study described a DNA microarray targeting the Centers for Disease Control and Prevention (CDC) category biothreat agents (Leski *et al.*, 2009), including arboviruses of the families *Arenaviridae*: *Lassa virus*, *Machupo virus*, *Guanarito virus* and *Junin virus*; and *Bunyaviridae*: genus *Hantavirus*: *Seoul virus*, *Hantaan virus*, *Puumala virus* and *Sin Nombre virus*. However, the RoboArboVirusChip described in this study includes probes for all the viruses mentioned in those previous studies and all arboviruses with sequences deposited in the GenBank, as we have mentioned above.

Dengue, the most important vector-borne virus disease, is caused by the *Dengue virus* (DENV), which belongs to the *Flavivirus* genus, *Flaviviridae* family. It has recently been estimated that approximately 390 million dengue infections occur per year, of which, 96 million manifest some level of disease severity (Bhatt *et al.*, 2013). Infection with any of the four viral serotypes (DENV-1 to -4) can be asymptomatic or lead to disease with clinical symptoms ranging from an undifferentiated and self-limiting fever to a severe dengue disease, which are mostly characterized by plasma leakage and hypovolemic shock that in some cases can be fatal (WHO, 2009). Secondary infection with a second virus serotype is a risk factor for the development of severe form of the disease (Halstead, 1970). Therefore, epidemiological surveillance is of great interest to the implementation of control of the spread of new introduced serotypes. The RoboArboVirusChip was able to clearly differentiate the four DENV serotypes, suggesting that it can be used in the surveillance of DENV serotypes circulation.

Co-infection with more than one virus type is a common event for arboviruses in hyper-endemic regions. Co-infection with more than one DENV serotypes and DENV/*Chikungunya virus* have been described in the literature (Figueiredo *et al.*, 2011; Sulca *et al.*, 2013; Cnops *et al.*, 2014; Parreira *et al.*, 2014). The microarray slide

described in this study was able to clearly identify viruses contained in three different mixtures: four DENV serotypes, four different viruses of the same genus (*Flavivirus* genus: ROCV, SLEV, YFV and DENV-2) and four viruses from different families (*Togaviridae* [MAYV], *Flaviviridae* [SLEV and BUSQV] and *Rhabdoviridae* [PIRYV]). Therefore, the RoboArboVirusChip could be used to identify co-infection with different viruses.

The RoboArboVirusChip was able to detect a minimum of 21-67 RNA copies/mL of DENV-1 to -4, which is similar to the sensitivity described by other DNA microarray platforms (Grubaugh, Mcmenamy, *et al.*, 2013; Huang *et al.*, 2013), and to the sensitivity of a real-time RT-PCR describes by our group and which has been used to detect DENV in serum and saliva samples (Dos Santos *et al.*, 2008; Poloni *et al.*, 2010; Muller *et al.*, 2014). These data suggest that the RoboArboVirusChip could be use to analyze clinical samples, which was confirmed by the detection of DENV in four clinical samples.

Finally, we have searched for roboviruses and other arboviruses in serum of 40 DENV negative patients but no virus was detected. May be other viruses different from roboviruses and arboviruses or even other pathogens, mimicking dengue symptoms, were the causative agents of the diseases in those patients, that is why we did not detected any virus with the RoboArboVirusChip. The dengue negative cases were collected during an epidemic outbreak in 2006 in Ribeirao Preto. The peak of dengue occurred between April and June, which coincided with the appearance of influenza cases that can be confused with dengue (CVE, 2006). In this sense, in a study performed in Puerto Rico in 2009, 31 of 289 dengue suspected cases was confirmed with dengue and most of the other were infected with influenza virus (n=136) (Zarocostas, 2009). Another study performed in Nicaragua in 2005-2009, analyzing 124 dengue suspected cases but negative by DNA microarray and new generation sequencing methods, detected mainly herpesviruses but not arbovirus or robovirus (Yozwiak *et al.*, 2012). Although there is circulation of other arboviruses during dengue outbreaks, this event seems to be not common (Mondini *et al.*, 2007; Terzian *et al.*, 2011).

7. CONCLUSIONS

1. A DNA microarray (RoboArboVirusChip) platform for detection of roboviruses and arboviruses at the species level was designed.
2. The RoboArboVirusChip successfully detected all reference viruses tested.
3. The chip was able to differentiate viruses in mixtures containing viruses of different families, same genus and different serotypes.
4. The chip showed sensitivity sufficient to detect viruses in clinical samples.
5. The RoboArboVirusChip was applicable to detect viruses in serum samples.
6. Permanent update of probes is needed since new sequences of viruses covered by the RoboArboVirusChip and sequences of new viruses are constantly been deposited in the GenBank.

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9. Appendix

Supplementary Table 1. Viruses and probes included in the RoboArbovirusChip.

Nº	Virus	Genera	Family	No. of Sequences	No. of Probes
1	Abu Hammad virus	Nairovirus	Bunyaviridae	1	5
2	Abu Mina virus	Nairovirus	Bunyaviridae	1	5
3	Adelaide River virus	Ephemerovirus	Rhabdoviridae	1	11
4	African horsesickness virus	Orbivirus	Reoviridae	57	124
5	African swine fever virus	Asfivirus	Asfarviridae	100	17
6	Aguacate virus	Phlebovirus	Bunyaviridae	3	15
7	Aino virus	Orthobunyavirus	Bunyaviridae	8	15
8	Akabane virus	Orthobunyavirus	Bunyaviridae	11	14
9	Alenquer virus	Phlebovirus	Bunyaviridae	3	19
10	Alkhurma hemorrhagic fever virus	Flavivirus	Flaviviridae	1	10
11	Allpahuayo virus	Arenavirus	Arenaviridae	3	10
12	Altai virus	Hantavirus	Bunyaviridae	1	5
13	Alto Paraguay hantavirus	Hantavirus	Bunyaviridae	2	10
14	Amapari virus	Arenavirus	Arenaviridae	2	10
15	Amur virus	Hantavirus	Bunyaviridae	12	15
16	ANAJ Hantavirus	Hantavirus	Bunyaviridae	1	4
17	Andes virus	Hantavirus	Bunyaviridae	3	16
18	Anhembi virus	Orthobunyavirus	Bunyaviridae	3	15
19	Anopheles A virus	Orthobunyavirus	Bunyaviridae	1	5
20	Anopheles B virus	Orthobunyavirus	Bunyaviridae	1	6
21	Apeu virus	Orthobunyavirus	Bunyaviridae	4	16
22	Apoi virus	Flavivirus	Flaviviridae	1	10
23	Araraquara virus	Hantavirus	Bunyaviridae	2	10
24	Araucaria virus	Hantavirus	Bunyaviridae	9	5
25	Arbia virus	Phlebovirus	Bunyaviridae	1	19
26	Arenavirus H	Arenavirus	Arenaviridae	2	7
27	Ariquemes virus	Phlebovirus	Bunyaviridae	3	15
28	Armero virus	Phlebovirus	Bunyaviridae	3	13

29	Aroa virus	Flavivirus	Flaviviridae	1	7
30	Artybash virus	Hantavirus	Bunyaviridae	2	9
31	Arumowot virus	Phlebovirus	Bunyaviridae	3	15
32	Asama virus	Hantavirus	Bunyaviridae	7	15
33	Ash River virus	Hantavirus	Bunyaviridae	2	10
34	Aura virus	Alphavirus	Togaviridae	1	10
35	Babanki virus	Alphavirus	Togaviridae	1	2
36	Bagaza virus	Flavivirus	Flaviviridae	2	9
37	Bandia virus	Nairovirus	Bunyaviridae	1	2
38	Banna virus	Seadornavirus	Reoviridae	17	38
39	Banzi virus	Flavivirus	Flaviviridae	1	10
40	Barmah Forest virus	Alphavirus	Togaviridae	1	9
41	Batai virus	Orthobunyavirus	Bunyaviridae	5	7
42	Batama virus	Orthobunyavirus	Bunyaviridae	1	2
43	Batu Cave virus	Flavivirus	Flaviviridae	1	3
44	Bayou virus	Hantavirus	Bunyaviridae	2	6
45	Bear Canyon virus	Arenavirus	Arenaviridae	3	9
46	Bebaru virus	Alphavirus	Togaviridae	1	2
47	Belterra virus	Phlebovirus	Bunyaviridae	3	11
48	Bermejo virus	Hantavirus	Bunyaviridae	2	10
49	Berrimah virus	Ephemerovirus	Rhabdoviridae	1	9
50	Bhanja virus	Phlebovirus	Bunyaviridae	3	12
51	Big brushy tank virus	Arenavirus	Arenaviridae	4	16
52	Birao virus	Orthobunyavirus	Bunyaviridae	2	10
53	Black Creek Canal virus	Hantavirus	Bunyaviridae	4	5
54	Black Mesa virus	Arenavirus	Arenaviridae	2	8
55	Blue River virus	Hantavirus	Bunyaviridae	1	5
56	Bluetongue virus	Orbivirus	Reoviridae	298	364
57	Boraceia virus	Orthobunyavirus	Bunyaviridae	1	4
58	Bouboui virus	Flavivirus	Flaviviridae	1	8
59	Bovine ephemeral fever virus	Ephemerovirus	Rhabdoviridae	2	10
60	Bozo virus	Orthobunyavirus	Bunyaviridae	2	4

61	Broadhaven virus	Orbivirus	Reoviridae	1	5
62	Buenaventura virus	Phlebovirus	Bunyaviridae	3	5
63	Buggy Creek virus	Alphavirus	Togaviridae	1	8
64	Bujaru virus	Phlebovirus	Bunyaviridae	1	2
65	Bukalasa bat virus	Flavivirus	Flaviviridae	1	1
66	Bunyamwera virus	Orthobunyavirus	Bunyaviridae	4	13
67	Bussuquara virus	Flavivirus	Flaviviridae	1	10
68	Bwamba virus	Orthobunyavirus	Bunyaviridae	1	2
69	Cabassou virus	Alphavirus	Togaviridae	2	1
70	Cacao virus	Phlebovirus	Bunyaviridae	1	2
71	Cache Valley virus	Orthobunyavirus	Bunyaviridae	7	14
72	Cachoeira Porteira virus	Orthobunyavirus	Bunyaviridae	3	14
73	Cacipacore virus	Flavivirus	Flaviviridae	3	10
74	California encephalitis virus	Orthobunyavirus	Bunyaviridae	4	11
75	Calovo virus	Orthobunyavirus	Bunyaviridae	1	5
76	Camp Ripley virus	Hantavirus	Bunyaviridae	6	11
77	Candiru virus	Phlebovirus	Bunyaviridae	3	8
78	Cano - Delgadito virus	Hantavirus	Bunyaviridae	2	7
79	Cao Bang virus	Hantavirus	Bunyaviridae	3	14
80	Carajas virus	Vesiculovirus	Rhabdoviridae	1	1
81	Caraparu virus	Orthobunyavirus	Bunyaviridae	4	12
82	Carey Island virus	Flavivirus	Flaviviridae	1	1
83	Carrizal virus	Hantavirus	Bunyaviridae	3	13
84	Castelo dos Sonhos virus	Hantavirus	Bunyaviridae	8	4
85	Catacamas virus	Hantavirus	Bunyaviridae	2	10
86	Catarina virus	Arenavirus	Arenaviridae	4	16
87	Catch-me-cave virus	Phlebovirus	Bunyaviridae	1	4
88	Cell fusing agent virus	Flavivirus	Flaviviridae	5	8
89	Chagres virus	Phlebovirus	Bunyaviridae	3	15
90	Chandipura virus	Vesiculovirus	Rhabdoviridae	2	4
91	Chandiru virus	Phlebovirus	Bunyaviridae	3	15
92	Chapare virus	Arenavirus	Arenaviridae	2	10

93	Chatanga virus	Orthobunyavirus	Bunyaviridae	8	16
94	Chikungunya virus	Alphavirus	Togaviridae	40	10
95	Choclo virus	Hantavirus	Bunyaviridae	3	18
96	Cholul virus	Orthobunyavirus	Bunyaviridae	3	4
97	Chuzan Virus	Orbivirus	Reoviridae	5	17
98	Cocal virus	Vesiculovirus	Rhabdoviridae	1	9
99	Colorado tick fever virus	Coltivirus	Reoviridae	6	23
100	Corfou virus	Phlebovirus	Bunyaviridae	3	6
101	Cowbone Ridge virus	Flavivirus	Flaviviridae	2	2
102	Crimean-Congo hemorrhagic fever virus	Nairovirus	Bunyaviridae	16	19
103	Cupixi virus	Arenavirus	Arenaviridae	3	9
104	Cy1014 virus	Flavivirus	Flaviviridae	1	1
105	D' Aguilar virus	Orbivirus	Reoviridae	2	5
106	Dakar bat virus	Flavivirus	Flaviviridae	2	2
107	Dandenong virus	Arenavirus	Arenaviridae	3	16
108	Deer tick virus	Flavivirus	Flaviviridae	2	2
109	Dengue virus type 1	Flavivirus	Flaviviridae	2	10
110	Dengue virus type 2	Flavivirus	Flaviviridae	2	10
111	Dengue virus type 3	Flavivirus	Flaviviridae	1	10
112	Dengue virus type 4	Flavivirus	Flaviviridae	9	10
113	Dobrava-Belgrade virus	Hantavirus	Bunyaviridae	7	16
114	Douglas virus	Orthobunyavirus	Bunyaviridae	3	12
115	Dugbe virus	Nairovirus	Bunyaviridae	5	16
116	Durania virus	Phlebovirus	Bunyaviridae	3	15
117	Eastern equine encephalitis virus	Alphavirus	Togaviridae	4	2
118	Echarte virus	Phlebovirus	Bunyaviridae	3	14
119	Edge Hill virus	Flavivirus	Flaviviridae	1	9
120	EgAN 1825-61 virus	Phlebovirus	Bunyaviridae	3	13
121	El Moro Canyon virus	Hantavirus	Bunyaviridae	2	9
122	Entebbe bat virus	Flavivirus	Flaviviridae	1	3
123	Eothenomys miletus Hantavirus	Hantavirus	Bunyaviridae	1	3
124	Epizootic hemorrhagic disease virus	Orbivirus	Reoviridae	35	125

125	Equine encephalosis virus	Orbivirus	Reoviridae	10	13
126	Erve virus	Nairovirus	Bunyaviridae	1	1
127	Eyach virus	Coltivirus	Reoviridae	6	18
128	Farallon virus	Nairovirus	Bunyaviridae	1	2
129	Flexal virus	Arenavirus	Arenaviridae	2	6
130	Fort Morgan virus	Alphavirus	Togaviridae	1	8
131	Fort Sherman virus	Orthobunyavirus	Bunyaviridae	3	13
132	Four Corners Hantavirus	Hantavirus	Bunyaviridae	3	4
133	Frijoles virus	Phlebovirus	Bunyaviridae	3	3
134	Gabek Forest virus	Phlebovirus	Bunyaviridae	1	4
135	Gadgets Gully virus	Flavivirus	Flaviviridae	2	2
136	Gbagroube virus	Arenavirus	Arenaviridae	5	12
137	Germiston virus	Orthobunyavirus	Bunyaviridae	2	10
138	Getah virus	Alphavirus	Togaviridae	36	5
139	Granada virus	Phlebovirus	Bunyaviridae	1	12
140	Great Island virus	Orbivirus	Reoviridae	3	15
141	Greek goat encephalitis virus	Flavivirus	Flaviviridae	1	8
142	Guanarito virus	Arenavirus	Arenaviridae	4	9
143	Guaroa virus	Orthobunyavirus	Bunyaviridae	3	12
144	Gumbo Limbo virus	Orthobunyavirus	Bunyaviridae	1	10
145	Hantaan virus	Hantavirus	Bunyaviridae	12	13
146	Hazara virus	Nairovirus	Bunyaviridae	3	16
147	Heartland virus	Phlebovirus	Bunyaviridae	6	15
148	Highlands J virus	Alphavirus	Togaviridae	29	7
149	HoJo virus	Hantavirus	Bunyaviridae	3	4
150	Hokkaido virus	Hantavirus	Bunyaviridae	1	5
151	Huitzilac virus	Hantavirus	Bunyaviridae	3	13
152	I612045 virus	Orthobunyavirus	Bunyaviridae	5	15
153	Iaco virus	Orthobunyavirus	Bunyaviridae	3	15
154	Icoaraci virus	Phlebovirus	Bunyaviridae	3	13
155	Iguape virus	Flavivirus	Flaviviridae	1	10
156	Ilesha virus	Orthobunyavirus	Bunyaviridae	3	11

157	Ilheus virus	Flavivirus	Flaviviridae	1	10
158	Imjin virus	Hantavirus	Bunyaviridae	5	10
159	Ingwavuma virus	Orthobunyavirus	Bunyaviridae	1	4
160	Inkoo virus	Orthobunyavirus	Bunyaviridae	4	12
161	Ippy virus	Arenavirus	Arenaviridae	2	11
162	Isfahan virus	Vesiculovirus	Rhabdoviridae	1	11
163	Isla Vista virus	Hantavirus	Bunyaviridae	3	7
164	Israel turkey meningoencephalomyelitis virus	Flavivirus	Flaviviridae	1	6
165	Itaituba virus	Phlebovirus	Bunyaviridae	3	9
166	Itaporanga virus	Phlebovirus	Bunyaviridae	2	6
167	Itaqui virus	Orthobunyavirus	Bunyaviridae	2	3
168	Ixcanal virus	Phlebovirus	Bunyaviridae	3	14
169	Jabora virus	Hantavirus	Bunyaviridae	3	5
170	Jacunda Virus	Phlebovirus	Bunyaviridae	3	10
171	Jamestown Canyon virus	Orthobunyavirus	Bunyaviridae	8	15
172	Japanese encephalitis virus	Flavivirus	Flaviviridae	2	10
173	Jeju virus	Hantavirus	Bunyaviridae	4	14
174	Jerry Slough virus	Orthobunyavirus	Bunyaviridae	3	11
175	Joa virus	Phlebovirus	Bunyaviridae	3	7
176	Jugra virus	Flavivirus	Flaviviridae	1	7
177	Junin virus	Arenavirus	Arenaviridae	4	15
178	Jurona virus	Vesiculovirus	Rhabdoviridae	1	10
179	Jutiapa virus	Flavivirus	Flaviviridae	1	2
180	Kadam virus	Flavivirus	Flaviviridae	1	3
181	Kadipiro virus	Orbivirus	Reoviridae	5	23
182	Kaeng Khoi virus	Orthobunyavirus	Bunyaviridae	1	5
183	Kairi virus	Orthobunyavirus	Bunyaviridae	3	10
184	Karimabad virus	Phlebovirus	Bunyaviridae	1	2
185	Karshi virus	Flavivirus	Flaviviridae	1	1
186	Kedougou virus	Flavivirus	Flaviviridae	1	3
187	Kemerovo virus	Orbivirus	Reoviridae	3	15
188	Kenkeme virus	Hantavirus	Bunyaviridae	3	14

189	Keystone virus	Orthobunyavirus	Bunyaviridae	2	7
190	Khabarovsk	Hantavirus	Bunyaviridae	1	5
191	Kimberley virus	Ephemerovirus	Rhabdoviridae	1	12
192	Klamath virus	Vesiculovirus	Rhabdoviridae	1	4
193	Kodoko virus	Arenavirus	Arenaviridae	2	5
194	Kokobera virus	Flavivirus	Flaviviridae	1	2
195	Koutango virus	Flavivirus	Flaviviridae	1	3
196	Kunjin virus	Flavivirus	Flaviviridae	1	3
197	Kupe virus	Nairovirus	Bunyaviridae	3	16
198	La Crosse virus	Orthobunyavirus	Bunyaviridae	9	16
199	Laguna Negra virus	Hantavirus	Bunyaviridae	20	10
200	Langat virus	Flavivirus	Flaviviridae	1	2
201	Lassa virus	Arenavirus	Arenaviridae	4	6
202	Latino virus	Arenavirus	Arenaviridae	2	7
203	Leanyer virus	Orthobunyavirus	Bunyaviridae	3	14
204	Lechiguanas virus	Hantavirus	Bunyaviridae	2	4
205	Liao ning virus	Seadornavirus	Reoviridae	3	22
206	Lipovnik virus	Orbivirus	Reoviridae	2	10
207	Lokern virus	Orthobunyavirus	Bunyaviridae	1	5
208	Louping ill virus	Flavivirus	Flaviviridae	1	1
209	Lujo virus	Arenavirus	Arenaviridae	6	14
210	Lumbo virus	Orthobunyavirus	Bunyaviridae	2	6
211	Luna virus	Arenavirus	Arenaviridae	6	16
212	Lunk virus	Arenavirus	Arenaviridae	4	15
213	Lymphocytic choriomeningitis virus	Arenavirus	Arenaviridae	2	16
214	M'Poko virus	Orthobunyavirus	Bunyaviridae	1	1
215	Macaua virus	Orthobunyavirus	Bunyaviridae	3	15
216	Machupo virus	Arenavirus	Arenaviridae	6	13
217	Maciel virus	Hantavirus	Bunyaviridae	2	9
218	Madrid virus	Orthobunyavirus	Bunyaviridae	1	2
219	Maguari virus	Orthobunyavirus	Bunyaviridae	4	8
220	Main Drain virus	Orthobunyavirus	Bunyaviridae	3	8

221	Malakal virus	Ephemerovirus	Rhabdoviridae	2	10
222	Maldonado virus	Phlebovirus	Bunyaviridae	3	15
223	Malpais Spring virus	Vesiculovirus	Rhabdoviridae	1	6
224	Maporal virus	Hantavirus	Bunyaviridae	3	14
225	Maraba virus	Vesiculovirus	Rhabdoviridae	1	14
226	Maripa Hantavirus	Hantavirus	Bunyaviridae	3	14
227	Marituba virus	Orthobunyavirus	Bunyaviridae	3	4
228	Massilia virus	Phlebovirus	Bunyaviridae	3	15
229	Mayaro virus	Alphavirus	Togaviridae	53	12
230	Meaban virus	Flavivirus	Flaviviridae	2	3
231	Melao virus	Orthobunyavirus	Bunyaviridae	4	9
232	Menekre virus	Arenavirus	Arenaviridae	4	13
233	Merino Walk virus	Arenavirus	Arenaviridae	2	15
234	Middelburg virus	Alphavirus	Togaviridae	2	2
235	Mobala virus	Arenavirus	Arenaviridae	2	14
236	Modoc virus	Flavivirus	Flaviviridae	2	2
237	Montana myotis leukoencephalitis virus	Flavivirus	Flaviviridae	1	5
238	Montano virus	Hantavirus	Bunyaviridae	20	15
239	Mopia virus	Arenavirus	Arenaviridae	7	12
240	Morogoro virus	Arenavirus	Arenaviridae	2	16
241	Morro Bay virus	Orthobunyavirus	Bunyaviridae	1	5
242	Morumbi virus	Phlebovirus	Bunyaviridae	3	9
243	Mount Elgon bat virus	Vesiculovirus	Rhabdoviridae	1	8
244	Mucambo virus	Alphavirus	Togaviridae	25	2
245	Mucura virus	Phlebovirus	Bunyaviridae	3	11
246	Muju virus	Hantavirus	Bunyaviridae	3	6
247	Muleshoe virus	Hantavirus	Bunyaviridae	1	
248	Munguba virus	Phlebovirus	Bunyaviridae	3	15
249	Murray Valley encephalitis virus	Flavivirus	Flaviviridae	2	4
250	Murutucu virus	Orthobunyavirus	Bunyaviridae	2	9
251	Nairobi sheep disease virus	Nairovirus	Bunyaviridae	5	10
252	Naranjal virus	Flavivirus	Flaviviridae	2	3

253	Ndumu virus	Alphavirus	Togaviridae	4	5
254	Neembucu Hantavirus	Hantavirus	Bunyaviridae	2	2
255	Nepuyo virus	Orthobunyavirus	Bunyaviridae	2	3
256	Newfound Gap Hantavirus	Hantavirus	Bunyaviridae	1	4
257	New York virus	Hantavirus	Bunyaviridae	2	6
258	Ngari virus	Orthobunyavirus	Bunyaviridae	3	2
259	Nique virus	Phlebovirus	Bunyaviridae	3	16
260	Nola virus	Orthobunyavirus	Bunyaviridae	1	1
261	North American arenavirus	Arenavirus	Arenaviridae	3	7
262	Northway virus	Orthobunyavirus	Bunyaviridae	3	9
263	Nova virus	Hantavirus	Bunyaviridae	2	10
264	Ntaya virus	Flavivirus	Flaviviridae	2	2
265	Nyabira virus	Orbivirus	Reoviridae	1	5
266	Nyando virus	Orthobunyavirus	Bunyaviridae	1	2
267	Odrenisrou virus	Phlebovirus	Bunyaviridae	3	15
268	Oliveros virus	Arenavirus	Arenaviridae	2	11
269	Omsk hemorrhagic fever virus	Flavivirus	Flaviviridae	2	2
270	Oran virus	Hantavirus	Bunyaviridae	2	5
271	Oriboca virus	Orthobunyavirus	Bunyaviridae	3	8
272	Oriximina virus	Phlebovirus	Bunyaviridae	3	14
273	Orogrande virus	Arenavirus	Arenaviridae	1	8
274	Oropouche virus	Orthobunyavirus	Bunyaviridae	22	24
275	Oxbow virus	Hantavirus	Bunyaviridae	3	14
276	Oyo virus	Orthobunyavirus	Bunyaviridae	3	11
277	Palma virus	Phlebovirus	Bunyaviridae	3	15
278	Pampa virus	Arenavirus	Arenaviridae	1	8
279	Paraná virus	Arenavirus	Arenaviridae	2	11
280	Pata virus	Orbivirus	Reoviridae	3	15
281	Peaton	Orthobunyavirus	Bunyaviridae	7	10
282	Pergamino virus	Hantavirus	Bunyaviridae	15	8
283	Perinet virus	Vesiculovirus	Rhabdoviridae	1	10
284	Phnom Penh bat virus	Flavivirus	Flaviviridae	2	4

285	Pichinde virus	Arenavírus	Arenaviridae	5	16
286	Pike fry rhabdovirus	Vesiculovirus	Rhabdoviridae	1	10
287	Piritai virus	Arenavírus	Arenaviridae	3	18
288	Piry virus	Vesiculovirus	Rhabdoviridae	3	10
289	Playa de Oro Hantavirus	Hantavírus	Bunyaviridae	3	8
290	Playas virus	Orthobunyavirus	Bunyaviridae	1	2
291	Pongola virus	Orthobunyavirus	Bunyaviridae	1	4
292	Potiskum virus	Flavivirus	Flaviviridae	1	2
293	Powassan virus	Flavivirus	Flaviviridae	1	2
294	Precarious point virus	Phlebovirus	Bunyaviridae	3	14
295	Prospect Hill virus	Hantavírus	Bunyaviridae	3	7
296	Punique virus	Phlebovirus	Bunyaviridae	3	10
297	Punta Salinas virus	Nairovirus	Bunyaviridae	1	2
298	Punta Toro virus	Phlebovirus	Bunyaviridae	4	18
299	Puumala virus	Hantavírus	Bunyaviridae	6	11
300	Qalyub virus	Nairovirus	Bunyaviridae	1	2
301	Qiandao Lake virus	Hantavírus	Bunyaviridae	3	8
302	Raza virus	Nairovirus	Bunyaviridae	1	2
303	Restan virus	Orthobunyavirus	Bunyaviridae	2	4
304	Rift Valley fever virus	Phlebovirus	Bunyaviridae	101	11
305	Rio Bravo virus	Flavivirus	Flaviviridae	1	2
306	Rio Grande virus	Phlebovirus	Bunyaviridae	1	4
307	Rio Mamore virus	Hantavírus	Bunyaviridae	3	15
308	Rio Negro virus	Alphavirus	Togaviridae	2	10
309	Rio Segundo virus	Hantavírus	Bunyaviridae	1	1
310	Rocio virus	Flavivirus	Flaviviridae	2	11
311	Rockport virus	Hantavírus	Bunyaviridae	7	15
312	Ross River virus	Alphavirus	Togaviridae	4	4
313	Royal Farm virus	Flavivirus	Flaviviridae	1	3
314	Saaremaa virus	Hantavírus	Bunyaviridae	4	13
315	Sabiá virus	Arenavírus	Arenaviridae	3	11
316	Saboya virus	Flavivirus	Flaviviridae	1	9

317	Sagiyama virus	Alphavirus	Togaviridae	2	1
318	Saint Croix River virus	Orbivirus	Reoviridae	2	8
319	Sal Vieja virus	Flavivirus	Flaviviridae	3	4
320	Salehabad virus	Phlebovirus	Bunyaviridae	1	3
321	Salobo virus	Phlebovirus	Bunyaviridae	3	9
322	San Angelo virus	Orthobunyavirus	Bunyaviridae	2	9
323	San Perlita virus	Flavivirus	Flaviviridae	1	1
324	Sandfly fever Naples virus	Phlebovirus	Bunyaviridae	5	7
325	Sandfly fever sicilian virus	Phlebovirus	Bunyaviridae	8	4
326	Sandfly Sicilian Turkey virus	Phlebovirus	Bunyaviridae	3	11
327	Sangassou virus	Hantavirus	Bunyaviridae	4	15
328	Sango virus	Orthobunyavirus	Bunyaviridae	2	10
329	Sapporo rat virus	Hantavirus	Bunyaviridae	2	2
330	Sathuperi virus	Orthobunyavirus	Bunyaviridae	3	8
331	Saumarez Reef virus	Flavivirus	Flaviviridae	1	1
332	Schmallenberg virus	Orthobunyavirus	Bunyaviridae	3	10
333	Seewis virus	Hantavirus	Bunyaviridae	3	12
334	Semliki Forest virus	Alphavirus	Togaviridae	11	5
335	Seoul virus	Hantavirus	Bunyaviridae	6	13
336	Sepik virus	Flavivirus	Flaviviridae	1	1
337	Serang virus	Hantavirus	Bunyaviridae	3	12
338	Serra do Navio virus	Orthobunyavirus	Bunyaviridae	3	10
339	Serra Norte virus	Phlebovirus	Bunyaviridae	3	14
340	Shamonda virus	Orthobunyavirus	Bunyaviridae	4	10
341	Shokwe virus	Orthobunyavirus	Bunyaviridae	2	2
342	Shuni virus	Orthobunyavirus	Bunyaviridae	1	4
343	Simbu virus	Orthobunyavirus	Bunyaviridae	1	1
344	Sin Nombre virus	Hantavirus	Bunyaviridae	3	7
345	Sindbis virus	Alphavirus	Togaviridae	60	7
346	Skinner Tank virus	Arenavirus	Arenaviridae	2	15
347	Snowshoe Hare virus	Orthobunyavirus	Bunyaviridae	3	7
348	Soochong virus	Hantavirus	Bunyaviridae	10	15

349	Sororoca virus	Orthobunyavirus	Bunyaviridae	3	14
350	South River virus	Orthobunyavirus	Bunyaviridae	3	8
351	Southern elephant seal virus	Alphavirus	Togaviridae	2	5
352	Spondweeni virus	Flavivirus	Flaviviridae	1	1
353	Spring viraemia of carp virus	Vesiculovirus	Rhabdoviridae	1	2
354	St. Louis encephalitis virus	Flavivirus	Flaviviridae	8	10
355	Stratford virus	Flavivirus	Flaviviridae	1	8
356	Tacaiuma virus	Orthobunyavirus	Bunyaviridae	1	2
357	Tacaribe virus	Arenavirus	Arenaviridae	2	14
358	Tahyna	Orthobunyavirus	Bunyaviridae	4	16
359	Tamana bat virus	Flavivirus	Flaviviridae	1	8
360	Tamiami virus	Arenavirus	Arenaviridae	2	13
361	Tanganya virus	Hantavirus	Bunyaviridae	2	10
362	Tehran virus	Phlebovirus	Bunyaviridae	3	7
363	Tembusu virus	Flavivirus	Flaviviridae	5	2
364	Tensaw virus	Orthobunyavirus	Bunyaviridae	3	5
365	Tete virus	Orthobunyavirus	Bunyaviridae	1	5
366	Thailand virus	Hantavirus	Bunyaviridae	8	7
367	Thottapalayam virus	Hantavirus	Bunyaviridae	3	10
368	Tick-borne encephalitis virus	Flavivirus	Flaviviridae	40	10
369	Tillamook virus	Nairovirus	Bunyaviridae	1	2
370	Tilligerry virus	Orbivirus	Reoviridae	3	15
371	Tinaroo virus	Orthobunyavirus	Bunyaviridae	3	9
372	Tlacotalpan virus	Orthobunyavirus	Bunyaviridae	2	1
373	Tonate virus	Alphavirus	Togaviridae	2	1
374	Tonto creek virus	Arenavirus	Arenaviridae	4	16
375	Topografov virus	Hantavirus	Bunyaviridae	3	14
376	Toscana virus	Phlebovirus	Bunyaviridae	7	11
377	Tribec virus	Orbivirus	Reoviridae	3	15
378	Trivittatus virus	Orthobunyavirus	Bunyaviridae	4	13
379	Trocaria virus	Alphavirus	Togaviridae	2	2
380	Tucunduba virus	Orthobunyavirus	Bunyaviridae	3	13

381	Tula virus	Hantavirus	Bunyaviridae	6	14
382	Tunari virus	Hantavirus	Bunyaviridae	3	6
383	Turkish sheep encephalitis virus	Flavivirus	Flaviviridae	1	8
384	Turuna virus	Phlebovirus	Bunyaviridae	3	17
385	Tyuleny virus	Flavivirus	Flaviviridae	1	1
386	Uganda S virus	Flavivirus	Flaviviridae	1	10
387	Umatilla virus	Orbivirus	Reoviridae	3	15
388	Umbre virus	Orthobunyavirus	Bunyaviridae	8	5
389	Una virus	Alphavirus	Togaviridae	1	1
390	Uriurana virus	Phlebovirus	Bunyaviridae	3	5
391	Ussuri virus	Hantavirus	Bunyaviridae	4	15
392	Usutu virus	Flavivirus	Flaviviridae	7	6
393	Utique virus	Phlebovirus	Bunyaviridae	3	3
394	Uukuniemi virus	Phlebovirus	Bunyaviridae	3	10
395	Venezuelan equine encephalitis virus	Alphavirus	Togaviridae	57	13
396	Vesicular stomatitis Alagoas virus	Vesiculovirus	Rhabdoviridae	1	10
397	Vesicular stomatitis Indiana virus	Vesiculovirus	Rhabdoviridae	1	2
398	Vesicular stomatitis New Jersey virus	Vesiculovirus	Rhabdoviridae	1	1
399	Vinces virus	Orthobunyavirus	Bunyaviridae	2	4
400	Weldona virus	Orthobunyavirus	Bunyaviridae	1	6
401	Wesselsbron virus	Flavivirus	Flaviviridae	1	3
402	West Nile virus	Flavivirus	Flaviviridae	3	10
403	Western equine encephalitis virus	Alphavirus	Togaviridae	36	2
404	Whitewater virus	Arenavirus	Arenaviridae	2	15
405	Wyeomyia virus	Orthobunyavirus	Bunyaviridae	3	9
406	Xingu virus	Orthobunyavirus	Bunyaviridae	2	6
407	Yaounde virus	Flavivirus	Flaviviridae	1	8
408	Yellow fever virus	Flavivirus	Flaviviridae	3	10
409	Yokose virus	Flavivirus	Flaviviridae	1	8
410	Yug Bogdanovac virus	Vesiculovirus	Rhabdoviridae	1	10
411	Yunnan Orbivirus	Orbivirus	Reoviridae	3	15
412	Zika virus	Flavivirus	Flaviviridae	1	11

Total	2144	4209
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