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Detection of Human arboviruses in the Northern Coast of Ecuador

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DEDICATORIA

A mis padres por todo su apoyo, por su dedicación, por siempre estar junto a mí, sin ellos no hubiera sido posible este logro.

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RESUMEN

Esta tesis consiste en dos artículos relacionados con la detección de arbovirus en la costa norte de Ecuador, el primero es el estudio de la incidencia de los cuatro serotipos de dengue (DEN-1, DEN-2, DEN-3 y DEN-4) en una comunidad rural localizada en la provincia de Esmeraldas, cerca de Colombia. Se analizó 434 muestras de suero de pacientes febriles del Hospital Civil de Borbón durante los años 2010 al 2014. El segundo artículo trata sobre la detección de tres arbovirus: Zika, Dengue y Chikungunya en muestras de suero recolectadas del Hospital Delfina Torres durante marzo-mayo del 2016 en la ciudad de Esmeraldas. Estas muestras fueron analizadas por serología y PCR tiempo real.

Palabras clave: Zika, Dengue, Esmeraldas, Ecuador, Borbón, aislamiento, Chikungunya

ABSTRACT

This thesis consists of two papers related to detection of the arboviruses in the northern Coast of Ecuador, the first one is the study of four dengue serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) in a rural community located in Esmeraldas province near Colombian border. Serum samples from febrile patients obtained at Civil Hospital of Borbon were analysed by retrotranscriptase PCR during the period of 2010-2014. The second paper describes the detection of three arboviruses, Zika, Dengue and Chikungunya in serum samples collected at the Hospital Delfina Torres from March to May 2016 in Esmeraldas city. These samples were analyzed by serology and real time PCR.

Key words: Zika, Dengue, Esmeraldas, Ecuador, Borbon, Dengue serotypes, virus

isolation, Chikungunya.

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PART I

SCIENTIFIC PAPER 1

Dengue Serotype Differences in Urban and Semi-rural Communities in Ecuador

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Key words: Dengue, Borbon, Rural community, urban community, Esmeraldas, Dengue serotypes.

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ABSTRACT

Dengue is a major vector-borne infection causing large outbreaks in urban communities in tropical regions. During the period 2010- 2014; 434 serum samples from febrile patients were collected from a semi-rural community hospital located in the norwestern region of Ecuador. Dengue virus (DENV) was investigated by reverse transcriptase PCR; a total of 48 samples were positive for dengue. During our study we detected DENV-2 and DENV-3 from 2010 to 2013 and the four DENV serotypes during the period 2013-2014. Surprisingly, our results contrasted with surveys carried out in urban centers throughout the Ecuadorian Coast in which DENV-1, DENV-2 and DENV-4 were prevalent during years 2010-2013 and only 2 serotypes (DENV-1 and DENV-2) in 2014.These results suggest that dengue viruses in semi-rural communities didn't originate in the Ecuadorian cities.

INTRODUCTION

Dengue is one of the most important vector borne diseases in many tropical and subtropical regions of the world (Kyle & Harris, 2008). The etiological agent is a flavivirus comprising 4 serotypes (DENV1-4) , which originated in Old World non-human primates 1000 years ago (Guzman, 2010). This virus is transmitted by 2 species of mosquitoes vectors: *Aedes aegypti* and *Aedes albopictus* which become infected when they feed on the blood of infected patients (Kraemer *et al*, 2015). Dengue virus has disseminated worldwide; in 2013 3.6 billion people were estimated to live in areas where dengue virus could be transmitted; 50 to 200 million dengue infections occur annually and 500,000 cases correspond to severe dengue which causes 20,000 deaths. Dengue has an endemo-epidemic pattern with outbreaks every 5 years. Between 2008 and 2012, more than 1.2 million people were infected, from these, 28,333 were severe cases (PAHO, 2014); in the Americas, 19% of the cases occurred in the Andean countries (WHO, 2009). Since dengue fever entered Ecuador in 1988 the number of cases has increased; in 2014 there were 15,446 cases reported, 67 were severe dengue, resulting in 11 fatalities (MSP, 2014). In 2015 there were 42,483 cases, 51 were severe dengue, no fatalities were reported (MSP, 2015).

The *Aedes* mosquitoes travel short distances (500 m) therefore transmission is facilitated by human mobility in urban centers where infected individuals live in close proximity to susceptible individuals (Muhammad *et al*, 2010). This is especially critical in developing countries where cities suffer from uncontrolled urban growth, poverty and lack of basic infrastructure such as water distribution and garbage management.

Water storage in open containers and accumulation of garbage favors the proliferation of mosquitoes (Padilla *et al*, 2012). Dengue in rural communities is thought to be the result of viral spillover from urban centers (Rabaa *et al*, 2013).

Control of dengue fever requires understanding the factors involved in the transmission of the virus. This report describes a discrepancy of serotype distribution between urban and semi-rural communities in Ecuador which may be important in future public health measures to control the disease.

MATERIALS AND METHODS

Sample collection

The study was conducted at the Civil Hospital in Borbon (HCB), in the Northwestern Coast of Ecuador. All the subjects accepted an oral informed consent which was approved by the USFQ Bioethics Committee of and University of Michigan Institutional Review Board.

A total of 434 serum samples from febrile patients were collected during the period of 2010-2014.

Viral RNA extraction

For viral ARN extraction, QIAamp Viral RNA Mini Kit was used with 140 µl of serum sample, according to manufacturer´s instructions. The serum sample was added to 560 µl of AVL (lysis buffer) wait 10 min and then 560 µl of ethanol 96% was added wait for 5

min. 640 µl were taken and put on a spin column centrifuge for 1 min at 4,651 X g, discard the residue of ethanol, add 500 µl of AW1 to the spin column centrifuge for 1 min at 4,651 X g , again discard the residue change collection tube and add 500 µl of AW2 centrifuge for 3 min at 12,281 X g , and again centrifuge for 1 min at 12,281 X g to dry the column. After that, 41 µl of elution buffer was added, centrifuge for 1 min at 4,651 X g, this step was repeated one more time. 82 µl of viral ARN was obtained and this was stored at -80°C until used. (Qiagen, USA)

Reverse transcriptase (RT-PCR) and sequencing

RT-PCR was performed with the SuperScript III One-Step RT-PCR and Taq DNA Polymerase System with 5 µl of viral RNA sample, according to manufacturer´s instructions. The primers designed amplify different amplicon sizes as follows: D1: 5´-TCA ATA TGC TGA AAC GCG CGA GAA ACC G, TS1: 5´-CGT CTC AGT GAT CCG GGG G (482 bp, D1-TS1), TS2: 5´-CGC CAC AAG GGC CAT GAA CAG (119 bp, D2-TS2), TS3: 5´-TAA CAT CAT CAT GAG ACA GAG C (290 bp, D3-TS3), TS4: 5´-TGT TGT CTT AAA CAA GAG AGG TC (389 BP, D4-TS4) (Harris *et al*, 1998). An electrophoresis was performed in a 1.5% of agarose gel with ethidium bromide. Furthermore, part of the PCR product was sent to Functional Biosciences, for sequencing.

Controls were kindly donated by Instituto Nacional de Investigación en Salud Pública (INSPI), Guayaquil-Ecuador. For testing the quality of RNA extracts β-actin gene amplification was used with the following primers: (primer forward, 5' CGG AAC CGC TCA TTG CC 3' and, reverse: 5' ACC CAC ACT GTG CCC ATC TA).

RESULTS

From 2010 to 2014, 11%(95% CI: 8.3-14.4) positive samples for dengue were obtained from semi-rural communities. From 2010 to 2013, DENV-3 was detected in semiurban communities but not in urban ones, while DEN4 was detected in urban but not in semi-rural communities (Table 1). From 2013 to 2014 all dengue serotypes were detected in semiurban communities but only DEN1 and DEN2 in urban communities (Table 2).

DISCUSSION

We found that semi-rural communities in the northern Coast of Ecuador (in close proximity to the Colombian border) have different dengue virus genotypes than urban coastal communities including the capital of the province, the port of Esmeraldas (table 2). In Colombia all the dengue serotypes have been reported since 2006 (Perez-Castro *et al*, 2016).

This phenomenon may indicate that people in these remote communities move more frequently to rural regions of the Colombian coast than to cities in Ecuador. There is no road connecting Ecuador and Colombia in this region, however there is an active commerce between semi-rural communities of these two countries which includes regular goods and illegal traffic of goods subsidized by the Ecuadorian government (such as gasoline and propane) and illegal drugs (Cragin &Hoffman, 2003).

The other possibility is sylvatic dengue transmission, although no report of sylvatic transmission of dengue virus has confirmed in the Americas. The Chocó humid forest had suffered massive deforestation, there are still some areas where howler monkeys

(*Alouatta palliata*) and the machin monkey (*Cebus albifrons aequatorialis*) (Freile & Vasquez, 2005) could be found. This means that there is the possibility that a sylvatic cycle is helping for the maintenance and transmission of the virus in a minor scale.

It is also important to indicate that the genotype discrepancies described in this manuscript could be due to technical problems due to differences in protocols used or inadequate sampling procedures. Unfortunately our study lacks information about demography , environmental conditions and clinical presentation. This report highlights the need for more research in this region. The potential entry of dengue virus (and other arboviruses) from Colombia to Ecuador by this route may require additional measures to control these diseases in Ecuador.

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Table 1. Dengue serotypes in a semi-rural community located in northern coast of Ecuador from 2010 to 2014

	SEROTYPES			
YEAR	**DENV-1**	**DENV-2**	**DENV-3**	**DENV-4**
2010-2011	0	5	1	0
2011-2013	0	3	2	0
2013-2014	20	7	8	2

Table 2. Comparison between the presence of different dengue serotypes in Ecuador in semi-rural and urban communities with serotypes in Colombia (Santander department).

	Years		
Communities	**2010-2011**	**2011-2013**	**2013-2014**
Serotypes in semi-rural communities	2, 3	2, 3	1, 2, 3, 4
Serotypes in Urban communities (Esmeraldas, Manabí, Guayas, El Oro, Zamora Chinchipe, Morona Santiago, Napo provinces)[1]	1, 2, 4[1]	1, 2, 4[1]	1, 2[1]
Serotypes detected in (Santander-Colombia)	1,2,3,4[2]	1,2,3,4[2]	1,2,3,4[2]

[1]INSPI, 2012

[2]Villabona-Arenas, 2016

SCIENTIFIC PAPER 2

First isolation, detection and full genome sequencing of Zika virus from febrile patient sera in Esmeraldas city, Ecuador.

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Key words: Zika, Arthropod-borne virus, Chikungunya, Flavivirus, Ecuador, MinION.

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ABSTRACT

Zika virus has been reported in 33 countries of the Americas since 2015. This member of the *Flaviviridae* has symptoms similar to dengue fever but it has been associated with microcephaly in newborns. Until August 2016 the Ecuadorian Ministry of Public Health (MSP) has reported a total of 1,877 confirmed cases and 899 suspected cases of Zika virus infection. Here we report the detection of this virus using reverse-transcriptase PCR and virus isolation in this region. Genomic analysis of 2 isolates suggested that these isolates were genetically related to Zika viruses isolated in Brazil.

INTRODUCTION

Zika is an arthropod-borne virus from the family *Flaviviridae*, genus *Flavivirus,* discovered in Uganda in 1947 (Petersen *et al.*, 2016). It has a single-stranded RNA genome with 10.7 nt in length and is transmitted from human to human by *Aedes aegypti* mosquitoes, but it has also non-vector borne transmission including transplacental, sexual and blood transfusion (Barzon *et al*., 2016). The symptoms of the disease are present on 20% of the infected people and these include rash, fever, arthralgia, conjunctivitis, vomiting and headache (Barzon *et al*., 2016).

In 2016 the World Health Organization (WHO) confirmed the association of Zika with microcephaly and Guillain-Barré syndrome and in consequence declared it as a global emergency (Kindhauser *et al*., 2016). Serological surveys showed that the virus was endemic of Africa and Asia where few cases were reported. The first large outbreak occurred in Yap, Micronesia in 2007 where 5,000 residents (70%) got infected (Petersen *et al.*, 2016). In March of 2015 it was identified for the first time in Brazil In this country 500,000 to 1,500,000 cases were reported from 2015 to February 2016 (Barzon *et al*., 2016). Since then, the virus spread rapidly through 33 countries in the Americas (Petersen *et al.*, 2016). In Ecuador the Ministry of Health reported the first two cases on January 2016 and in August , Ecuador's Ministry of Health reported a total of 1,877 confirmed positive Zika cases and 899 suspected cases of Zika. Moreover 161 cases were confirmed in pregnant women. In Esmeraldas province; 152 positive cases for Zika were reported, from these 89 (58,6%) of the cases were reported in Esmeraldas city, 53 (34,9%) in San Lorenzo, 9 (5.9%) in Quinindé and 1 (0,7%) in Río Verde; 17 cases were from pregnant women (MSP, 2016).

In this study we report the presence of Zika virus infection in febrile patients from Esmeraldas province from March to May 2016 using real time PCR. Additionally we report the first isolation of the Zika virus in Ecuador.

MATERIALS AND METHODS

Sample collection

For this study , 211 samples were collected from febrile patients at Hospital Delfina Torres de Concha located in Esmeraldas city from March to May 2016. All patients previously accepted an oral consent approved by USFQ Bioethics Committee. Patient serum was obtained from venous blood samples and immediately stored in liquid nitrogen then transported to USFQ Virology laboratory for the analysis.

Differential diagnosis by IgM ELISA

An initial IgM ELISA differential diagnosis dengue fever was done for 45 serum samples at the hospital laboratory according to manufacturer´s instructions (Panbio, USA). These 45 samples were selected without any epidemiological criterium..

Differential diagnosis by real time PCR

From 140 µl of all sera samples, 80 µl of total RNA was extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen Gmbl, Germany).

The real-time PCR multiplex kit (Dengue, Zika and Chikungunya Genesig easy kit, UK) was used for preparing the following reaction; 10 µl of Oasig OneStep Mastermix, 1µl of

primer/probe mix, 1 µl of internal control, 1 µl of water and 4 µl of sample RNA, to a final volume of 20 µl was prepared. The following thermocycling conditions were used: Reverse transcription 55°C for 10 min, reverse transcriptase activation 95°C for 2 min followed by 50 cycles of denaturation at 95°C for 10 sec and 60°C for 60 sec. Primer sequences were not provided by manufacturer.

Isolation of Zika virus from patient sera

For Zika virus isolation a dilution 1: 10 of serum sample was made as follows: 30 µl of serum sample with 270 µl of 2% FBS of Leibovit'z media (L-15, Gibco, UK), added to a monolayer of C6/36 (*Aedes albopictus*) cell line in T25 tissue culture flasks (Falcon, USA). Flasks were gently rocked every 10 min for one hour to ensure monolayers were covered. 10 ml of 5% FBS- enriched L-15 media was added and cells were incubated for 7 days at 28°C.

Real time PCR was carried out using an individual Zika virus kit (Genesig, UK) to test for virus growth. Virus was then harvested by transferring supernatant to 15ml sterile tubes (Falcon, USA) that were then centrifuge for 2,325 *X g* for 5 min to remove cellular debris. The supernatant was enriched with 20% Fetal Bovine Serum and transferred to 1ml cryovials (NUNC, USA) and stored at -80°C.

Sequencing of isolated Zika virus strains and Phylogenetic analysis of Zika (Ec_Es_0416_062 & EC_Es_0416_089)

Sequencing was made using the Nanopore MinION technology. MAFFT program was used for the phylogenetic analysis, the two Zika virus genome sequences from Ecuador

were aligned together with all published and available near-complete ZIKV genomes and longer sub-genomic regions (>1500nt) of the Asian genotype as of early September of 2016. Maximum likelihood (ML) and Bayesian phylogenetic inferences were performed using the PhyML and BEASTv1.8.2 programs, respectively. The ML phylogeny was reconstructed using the best-fitted general time reversible nucleotide substitution model with a proportion of invariant sites (GTR+I) as determined by jModelTest2. Statistical support for phylogenetic nodes was assessed using a bootstrap approach (with 100 replicates). A Bayesian molecular clock phylogeny was estimated using the best fitting evolutionary model determined by Bayesian model selection in Faria, *et al*; specifically, a GTR+I substitution model with a strict molecular clock, a Bayesian skyline coalescent prior and a non-informative CTMC reference prior for the molecular clock rate.

RESULTS

Molecular and serological analysis

From 211 samples, 22 samples (10,42% (CI: 6.7-15.4)) were positive for Zika by real time PCR. All samples were negative for Chikungunya, Dengue and Leptospira. No coinfections were found. During this period of time, from 211 only 45 samples were analysed with IgM ELISA, from these, 8 (17,77%) serum samples were IgM positive for Dengue.

Viral isolation

Virus was successfully isolated from two Zika positive sera samples for which initial Ct values were 26, 5 and 24,17 and after seven days of growth in the c636 cell line a higher virus production was evident with Ct values of 22,3 and 15,53 respectively. No cytophatic effect was observed in C6/36 cells. Both patients presented with fever for 2 and 3 days respectively, while only patient 1 reported nausea, myalgia and arthralgia. Two haematology analysis parameters were common in both patients; a high percentage of neutrophils (72,9%; 67,1%, respectively) and a high percentage of lymphocytes (14,9%; 21,5%, respectively) as shown in annexes table 1.

Phylogenetic results

The sequences are already in the Genebank with accesion number (KX879603, KX879604) respectively. The two Zika virus genomes from Ecuador clustered together with a sequence isolated from the Paraiba state in Brazil (Accession number: KX280026, posterior probability 0.96, bootstrap support 75%, see Figure 2).

DISCUSSION

We detected Zika RNA in 22 (10.4%) of 211 serum samples from febrile patients in Esmeraldas city, and weren't able to amplify nucleic acids of dengue , chikungunya or *Leptospira*. However we detected anti-dengue IgM antibodies in 17% of 45 sera analyzed by PCR. It is worth noticing that anti-dengue IgM could persist for 2-6 months after infection (Prince & Matud, 2011). Higher incidence of Zika infection than dengue

or chikungunya infections may be caused by different susceptibility of the Ecuadorian population to these viruses at the time of our study (Chouin Carneiro *et al*., 2016). The Ecuadorian population has some level of immunity to dengue and chikungunya viruses because these viruses have been present in Ecuador since 1988 and 2014 respectively. Additionally lack of antibodies against Zika viruses may cause greater rates of fetal infection and birth defects (Zhao *et al*., 2016). Although this idea has been challenged (Tabata *et al*., 2016) further, Ecuador has no reported cases of microcephaly caused by Zika virus.

We also isolated Zika virus from 2 febrile patients, surprisingly the nucleotide sequences of these isolates had higher sequence similarity to Brazilian isolates than sequences from Colombian isolates. The northern Ecuadorian Coast has social and commercial interactions with Colombia, and to our knowledge it has little interaction with Brazil. In the last 8 years Zika spread fast to different parts of the world possibly due to international travel and urbanization (Zhu *et al*, 2016).

Ecuadorian population is still susceptible to Zika virus; The prevalence of Zika virus in serum samples was 10 %. Molecular techiques were important for the diagnosis, since no serology was made. The successful isolation of the virus might be useful for other investigations about pathogenicity. New control measures are needed within Latin American countries to prevent the circulation and potential transmission of the virus.

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Disclaimer: The authors declare no conflict of interest.

Supplementary materials: ZIBRA and Genbank accession numbers

Ethical approval: Use of patient samples that provided oral consent was included as part of an approved ongoing Dengue study between USFQ and University of Michigan with number 2010-17

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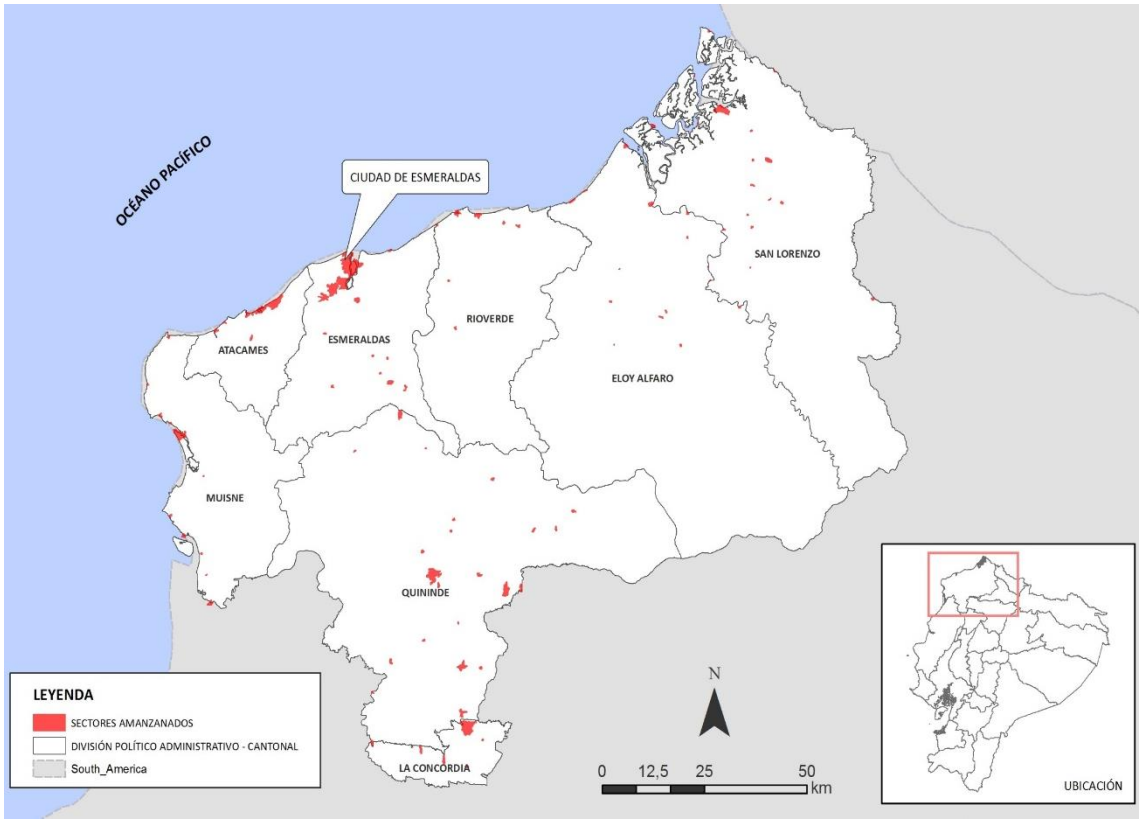

Figure 1. Esmeraldas city within Esmeraldas province on the North West coast of Ecuador.

Figure 2. Phylogenetic alignment of Zika isolates EC_Es_0416_062 & EC_Es_0416_089 with Bayesian and Maximun likelihood methods and analysed with 100 replicates for bootstrap testing.

PART II

ANNEXES

Tabla 1.Clinical parameters of two patients positive for Zika virus.

	Reference values	Patient ID 062	Patient ID 089
Days of Fever	n/a	2	3
Myalgia	n/a	Y	n
Arthralgia	n/a	Y	n
Nausea	n/a	Y	n
Sex	n/a	M	m
Age	n/a	27	31
Leukocytes	5-10 x10^3/µL	7,03	4,34
Neutrophils%	46-62 %	72,9	67,1
Lymphocytes %	0,80-4 %	14,9	21,5
Monocytes %	2-8 %	8,9	9,6
Eosinophils %	1-6 %	2,8	1,5
Basophils %	0-1%	0.5	0,3
Neutrophils #	2-7 x10^3/µL	5,13	2,92
Lymphocytes #	0,8-4 x10^3/µL	1,05	0,94
Monocytes #	0,12-1,2 x10^3/µL	0,62	0,41
Eosinophils #	0,02-0,5 x10^3/µL	0,2	0,06
Basophils #	0-0,1 x10^3/µL	0,03	0,01
Erithrocytes	4,3-5,70 x10^6/µL	5,31	4,71
Hemoglobin	13,20-17,80 g/dL	15,4	14,2
Hematocrit	40-54 %	46,7	43,6
Platelets	150-450	233	180