USDEP Ultra-Sensitive Diagnosis for Emerging Pathogens

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CAPTURE AND ENRICHMENT OF EMERGING PATHOGENS FOR MULTIPLE AND ULTRA-SENSITIVE DIAGNOSTIC (USDEP)

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Final Activity Report

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PROJECT SUMMARY

USDEP: « CAPTURE AND ENRICHMENT OF EMERGING PATHOGENS FOR MULTIPLE AND ULTRA-SENSITIVE DIAGNOSTIC »

USDEP was a specific targeted research project (STREP) funded by the 6th Framework Programme in the area of **LSH-2005-1.2.2-4: Development of new diagnostics** and dedicated to SMEs. Its specific goal was to promote development of new tools and techniques for early diagnosis of disease, for monitoring disease progression and for guiding therapeutic interventions. The USDEP project was started in November 2006 and was terminated in April 2010.

Despite remarkable advances in medical research and treatments during the 20th century, infectious diseases remain among the leading causes of death worldwide and new infectious diseases continue to evolve and emerge In the 1970's WHO proclaimed that eradication of smallpox should be attempted. This goal has been achieved 1979. There is now consensus that the list of newly emerging or re-emerging pathogens is continuously growing. During the last decades pathogens such as Marburg, Ebola, Hepatitis-C and Hantavirus, HIV and more recently, SARS coronavirus have emerged. Today the risk of a new influenza pandemic again highlights the global threat of infectious diseases. In addition, the possibility of bioterrorist attacks using highly pathogenic viruses and bacteria cannot be ignored. Furthermore, the requirements for sensitivity and specificity of diagnostics have increased. Therefore adopting the appropriate containment measures towards emergent pathogens, fast, sensitive and reliable diagnostics are a key element. Nucleic acid amplification is widely used for the detection and identification of pathogens. However, despite a gain in sensitivity brought by these methods, the European Quality Assurance (EQA) studies revealed that molecular diagnostics still showed a poor overall test proficiency compared to serological diagnostics and one of the main problems for pathogen detection in clinical but also in environmental samples is that they generate false negative results.

False negative results (clinical and environmental samples undetectable with current diagnostic methods) are a major obstacle for the diagnosis of infection. This is mainly due to the lack of a rapid and reliable pathogen concentration methodology, and the inability of most of the currently used technologies to eliminate or neutralize interfering "natural inhibitors" present in samples.
While studying the interactions between viruses and plasma proteins and the interactions between conserved molecular structures of viruses and

host cells, we identified the human protein apolipoprotein H (ApoH) as a protein that strongly interact with different non-self elements including viruses, bacteria and parasites and has the appropriated characteristics to be considered as a candidate for the development of novel capture technologies. ApoH, also known as b2-glycoprotein I (b2-GPI), was originally identified in plasma as a perchloric acid glycoprotein. This protein has a molecular mass of 50 kDa and its plasmatic concentration is close to 200 µg/ml. The described amino-acid sequence showed an unusual composition for a plasma protein, with 6.2 % cysteine and 8.3 % proline. This protein is composed of 5 segments of 60-80 amino acid residues, each containing the consensus sequence of the complement control protein (CCP) module, which implies that ApoH is a member of CCP superfamily. According to some reports, about 30% of ApoH is associated with the plasma lipoprotein fraction.

ApoH-technology was successfully tested on clinical samples from patients infected with numerous viruses: validation of ApoH technology was performed via employing combination of pathogen concentration step using ApoH with well characterized and pre-validated diagnostic platforms. ApoH technology was also successful for bacterial capture and concentration: Staphylococcus*, Escherichia coli* and other nosocomial bacteria.

Clinical validation was particularly successful for detection of ANDES Hantavirus and enabled efficient identification of clinical cases (published in Journal of Virology, May 2009, p. 5046-5055, Vol. 83, No. 10).
Promising results have also been obtained for HCV, Dengue virus, HIV and HBV. USDEP data suggest that ApoH-coated beads may be useful in reducing the number of samples undetected (false negatives) using current or improved molecular (PCR) or immune (ELISA) techniques, probably by eliminating natural inhibitors.
Detection of mosquito transmitted infectious diseases was demonstrated in multiplex systems which is likely to allow the improvement of fever diagnosis when introduced commercially (presented at the 19th Annual Meeting of the German Virology Society in Leipzig, Germany, March 2009; the 14th International Congress on Infectious Diseases, Miami, USA, March 2010 and the 4th European Congress of Virology, Lake Como, Italy, April 2010).
Real-time RT-PCR and one-step RT-PCR protocols were developed to detect hepatitis viruses in HIV co-infected patients (single and multiplex) as well as generic flavivirus, chikungunya virus (ChikV) and malaria parasites (in triplex system).
Efficient detection of Human Endogenous Retrovirus family W (HERV-W) envelope and capsid antigen was demonstrated using ApoH based ELISA in serum of patients with neurologic diseases such as multiple sclerosis

and schizophrenia (published in Biol Psychiatry. Dec 2008; 64(12): p. 1019-23).

Novel tools, using ApoH technology were developed and particularly validated on clinical samples:
1) **Mini-Array Detection Kit of DENV serotypes** (DEN-1, -2, -3, -4), have been validated with samples from Dengue infected patients, collected in Guyana, Cambodia and St Martin,
2) **Mini-Array Detection Kit** for the diagnosis of **6 mosquito-transmitted pathogens in humans** has been developed: including Yellow Fiver Virus (YFV), St Louis Encephalitis Virus (SLEV), Japanese Encephalitis Virus (JEV), West Nile Virus (WNV), ChikV and malaria parasites,
3) **Mini-Array Detection Kit** prototype has been designed for Hantavirus detection and differentiation (Andes, Puumala, Seoul and Sin Nombre),
4) **e-Biochip microarray for HIV, HBV, HCV, HEV** has been produced and validated, using clinical samples with HCV and HIV from Chile, Italy and St Martin,
5) **Mosquito e-Biochip** for the simultaneous detection of DENV, YFV, JEV, WNV, ChikV, malaria parasites has been validated using clinical samples (dengue and malaria) from Cambodia, Guyana and St Martin,
6) **Rotavirus ApoH-ELISA** have been established and validated with Rota A Virus positive stool samples provided by the German National Reference Centre for Enteroviruses,
7) **ANDES ApoH-ELISA** was developed, validated and established in clinical setting to detect ANDES Hantavirus from urine of patients in early stage of the disease.

USDEP has provided a range of innovative tools to:

- **capture a wide range of pathogens for diagnostics or functional purposes**
- **concentrate pathogens from large sample volumes of complex biological mixtures**

ApoH technology may be applicable as a simple and cost effective step for treatment of samples in ultrasensitive detection of pathogens in transplantation, blood banks, in clinical management and in detection of environmental pathogen contamination.

USDEP project has significantly contributed to the competiveness of European SMEs via engaging and supporting five SMEs: ApoH Technologies, AJ eBiochip, GenExpress, ImmunoClin and SKULDTECH. In collaboration with Robert Koch-Institut (RKI), Frauenhofer-Institut für Siliziumtechnologie (ISIT) and the Institut de Recherches pour le Développement (IRD) the SMEs were able to increase their scope of activities and to create long term collaborative relationship.

OBJECTIVES OF USDEP PROJECT

One of the main concerns for pathogen detection in clinical, but also in environmental samples, is that they generate false negative results that are mainly due to: (i) presence of inhibitors, (ii) absence of suitable molecular detection methods (e.g. PCR) and (iii) lack of a rapid and reliable pathogen concentration methodology.

The aim of USDEP project was the use of ApoH-coated solid matrix as a generic capture method for a wide range of pathogens in order to concentrate them from large sample volumes of complex biological mixtures, otherwise poorly amenable for molecular methods and thus to improve their detection threshold.

During the first and second project year, tools and protocols were developed using prototype viruses, bacteria and standard diagnostic methods. The tested microorganisms included:

- (i) enveloped and non-enveloped, small and large, DNA and RNA viruses of Public Health importance such as HIV, HBV, HCV, RVFV, Orthopoxvirus, Dengue Virus, Marburg Virus, respiratory virus (H1N1, H3N2, FluA, FluB, RSV, Adenovirus)
- (ii) gram (+) and gram (−) bacteria such as *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Streptococcus pyogenes, Acinetobacter baumanii , Propionibacterium acnes, Mycobacterium abscessus, Nocardia farcinica*
- (iii) malaria parasite: *Plasmodium falciparum*.

Furthermore, methods for sample preparation were established for different types of specimen, saliva, plasma, serum, urine and faeces as well as various environmental samples. Immunological (ELISA) and molecular (real-time RT-PCR, Mini-Array and electrical Biochip-Microarray) detection techniques, either derived from published protocols or developed or optimized during this study.

During the third year, different generations of ApoH-coated matrices were evaluated, in blind, for the pre-treatment of clinical samples, notably from patients with suspected viral (HCV, Dengue virus, ANDES Hantavirus, HBV, HIV, HERV) or bacterial infections with negative diagnostics (such as non-cultivable bacteria). ApoH-coated matrices were also evaluated for the diagnostic of dengue virus and rotavirus from environmental samples (mosquitoes, faeces). The results were compared with available clinical data and showed that the efficiency of detection after ApoH-capture in almost all cases was superior or equivalent to that obtained in absence of ApoH step.

The overall obtained results have demonstrated that the objectives of the USDEP project have been achieved and resulted in the development of a novel generic tool that permits to:

- capture a wide range of pathogens for diagnostics or functional (test of their infectivity) purposes,
- concentrate pathogens from large volumes of complex biological samples,
- improve pathogen detection threshold after integration of this novel technology in real-time RT-PCR, biochips or Mini-Array molecular detection techniques.

USDEP PROJECT SUCCESS
ENHANCEMENT OF PATHOGEN DETECTION IN CLINICAL SAMPLES USING THE APOH PRE-TREATMENT STEP

ApoH-capture and ANDES Hantavirus detection

Hantavirus cardiopulmonary syndrome (HCPS) is a highly pathogenic emerging disease (40% case fatality rate) caused by New World hemorrhagic Hantaviruses. Hantavirus infections are transmitted to humans mainly by inhalation of virus-contaminated aerosol particles of rodent excreta and secretions. At present, there are no antiviral drugs or immunotherapeutic agents available for the treatment of hantaviral infection, and the survival rates for infected patients hinge largely on early virus recognition and hospital admission and aggressive pulmonary and hemodynamic support. USDEP project has shown that ApoH-coated magnetic beads or ApoH-coated enzyme-linked immunosorbent assay plates capture and concentrate the Andes virus (ANDV) from complex biological mixtures, such as serum and urine of infected patients, allowing to detect it by both, immunological and molecular approaches.

Furthermore, a high throughput-screening assay for ANDV has been developed and **urine** samples from acute-hantavirus cardiopulmonary syndrome patients (44 out of 50 tested patients) have been tested. These results were presented **in two scientific meetings** and have been published in Journal of Virology, May 2009, p. 5046-5055, Vol. 83, No. 10. Moreover and strikingly, we have also demonstrated that ApoH-coated matrices were able to capture Hantavirus and enhance their detection, **particularly in samples from false negative diagnosed patients.**

- Molecular Approach to detect ANDV Hantavirus from Clinical samples

Urine and serum samples were tested for the presence of ADNV using ApoH concentration step. Detection was done with specific qPCR (Table 1) or a RT-PCR/heminested PCR (Fig. 1).

Table 1. ANDV Quantitative PCR applied to urine and serum in the presence or in the absence of ApoH

Virus load Real-Time PCR			
Sample	Origin	Roche ™ diagnostic	ApoH ™ magnetic beads improvement
Negative	Urine	NEG	NEG
Negative	Urine	NEG	NEG
Negative	Urine	NEG	NEG
Negative	Serum	NEG	NEG
RGL2	Urine	33177	33715
RGL3	Urine	114945	2751
RGL5	Urine	65628	53463
RCC1	Urine	423216	13743
RCC2	Urine	54205	11914
RCC3	Urine	672815	22555
RCC4	Urine	**NEG**	**22602**
RCC5	Urine	4766	2580
ROMEO	**Serum**	**NEG**	**37668**

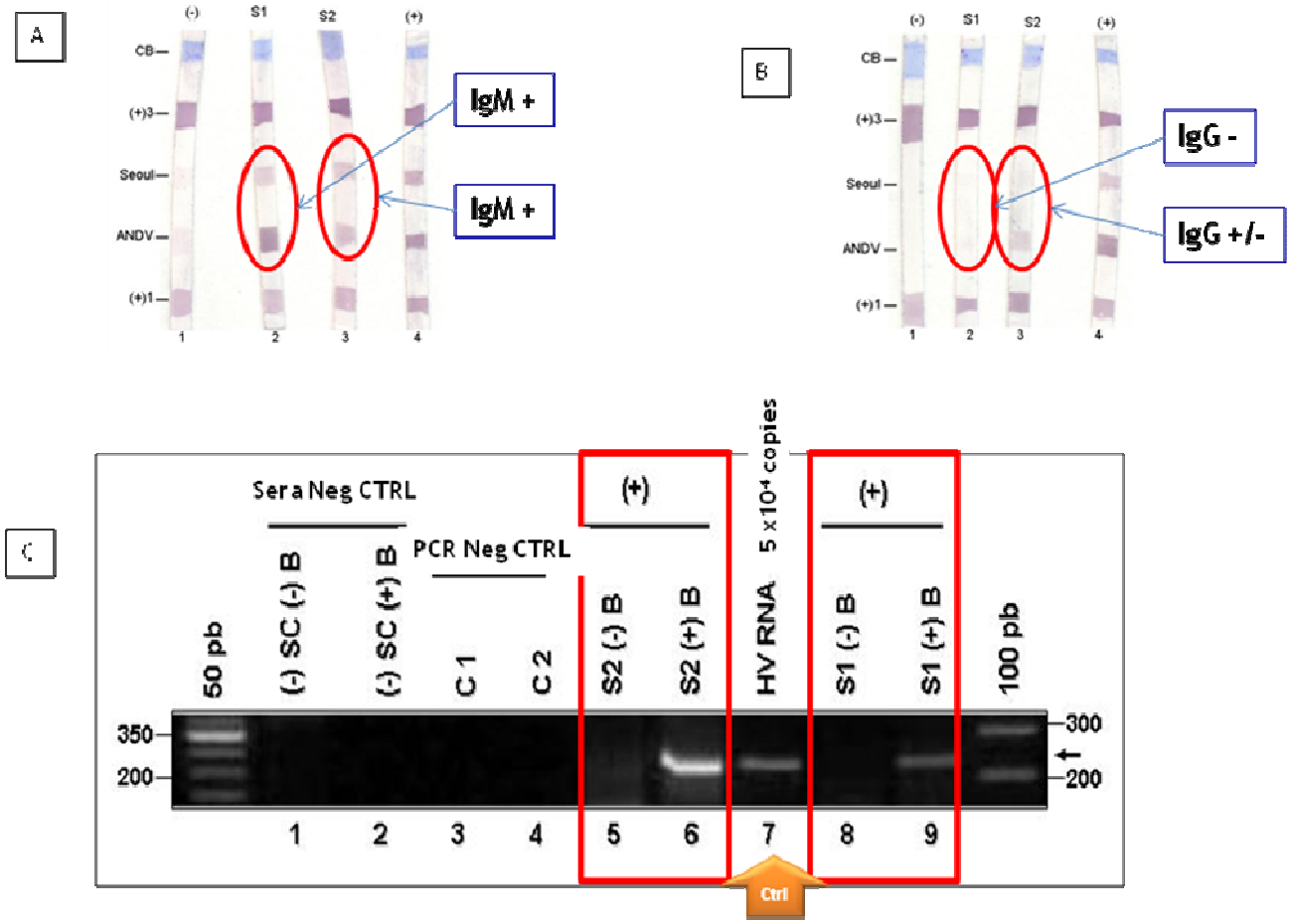


Figure 1: ANDV binds serum-purified human ApoH. Serum from patients S1 and S2 were tested for the presence of anti-hantavirus IgM (A) and IgG (B) antibodies by SIA using affinity-purified recombinant ANDV and Seoul virus N antigen. As internal controls, two levels (+1 and +3) of human IgG were applied to the blot. Serum from a negative donor (-) and of from a known HCPS-positive patient (+) were used as controls. Coomassie blue (CB) was used to determine the orientation of the strip. (C) Serum samples from patients S1 and S2 were divided in two. RNA was directly extracted from 300 µL of sample one without the addition of ApoH beads [(-) B; lanes 5 and 8]. In parallel, the second sample of each patient was submitted to an additional step of ANDV capture by ApoH-coated magnetic beads prior to RNA extraction [(+) B; lanes 6 and 9]. Extracted RNA was used as the template in a RT-PCR/heminested PCR assay. As an additional control, total RNA extracted from sera of healthy negative donors with [(-) SC (+) B; lane 2] or without [(-) SC (-) B; lane 1] a step of virus capture and concentration by ApoH-coated magnetic beads was used as the negative control. Water controls for the RT-PCR (C1; lane 3) and heminested PCR (C2; lane 4) were included. In vitro-transcribed RNA corresponding to the ANDV S segment was used as a positive control for RT-PCR (HV RNA; lane 7). The arrow indicates the expected amplicon.

ApoH-coated beads help to identify ANDV Hantavirus infections from serum of suspected infected patients at the early phases of infection.

The ApoH-capture of Dengue virus (DENV) in clinical samples from negative diagnosed Cambodian patients declared as "indeterminant or suspected" for Dengue Virus infection

Dengue virus (four subtypes) is a mosquito-borne remerging infection that has become a major global public health concern, for which the hallmark is the Dengue hemorrhagic fever.
In recent decades the incidence of dengue has grown dramatically around the world. Some 2.5 billion people – two fifths of the world's population – are now at risk from dengue. WHO currently estimates there may be 50 million dengue infections worldwide every year.
In order to assess the ability of ApoH to solve the diagnostic problem of suspected patients, clinical samples were obtained from Cambodia and tested in the absence and in the presence of two generations of ApoH beads.
Figure 2 shows that the ApoH pre-treatment associated to RT-qPCR, developed by the USDEP consortium, allow turning positive (>50%) many previous suspected samples (14/24) that clearly previously were **false negatives**. Some suspected samples remain negatives confirming that probably they are really DENV negatives but that may be infected by another virus.

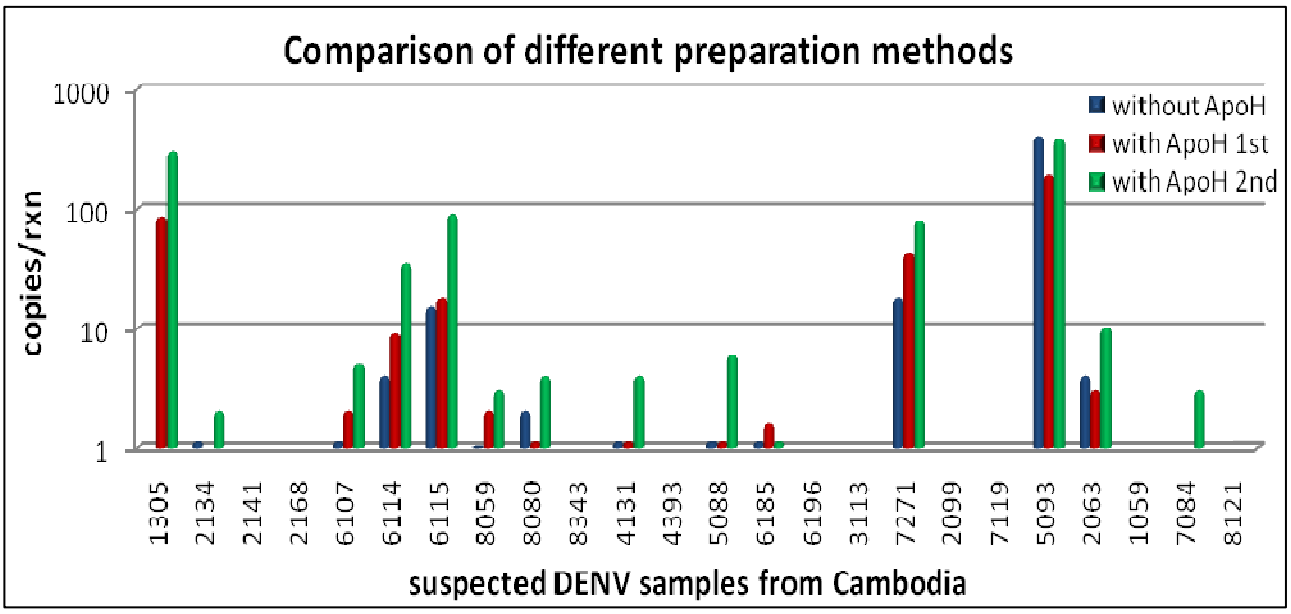


Figure 2: DENV detection in serum samples from Cambodia DENV suspected patients with or without ApoH (1st and 2nd generation beads) pre-treatment.

These Cambodia samples demonstrate that the ApoH-DENV capture **strikingly improve sensitivity of Dengue Virus detection**, thus, strongly reducing the number of false negative diagnostics.

Mini-Array were also developed to detect and differentiate in a single reaction the 4 dengue subtypes (Fig. 3).

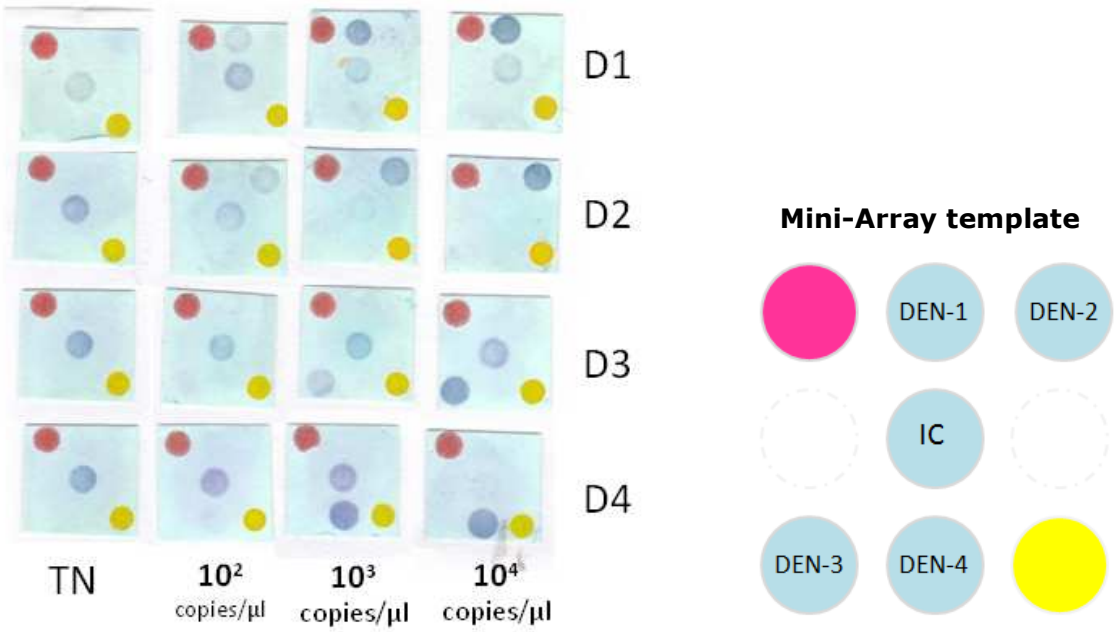


Figure 3: Mini-Array detection of 4 dengue subtypes with Mini-Array membrane interpretation template.

The results in figure 3 show hybridized membranes able to detect in one shoot 4 dengue subtypes. The above picture shows a serial dilution for the 4 dengue subtypes. This Mini-Array Dengue kit has several advantages which are crucial for virus detection: rapidity (only 4 - 5 hours), sensitivity, high specificity, one-step detection of multiplex infections, and interpretation of the results easy and done with the naked eye, **easy to use**.

The ApoH-capture of HIV in clinical samples from Argentina

ApoH pre-treatment showed enhancement for the majority of Argentinean samples (20/26) (Fig. 4). Some samples which were negative (5/26), without ApoH became positive with ApoH (including two samples that were also below the detection limit at the hospital). The lower values with ApoH (3/26) could be due to the fact that we used a lower volume of sample as compared with what is usually used in hospitals (a minimum of 1 mL of plasma), and/or to the imperfect shipment from Argentina to Europe.

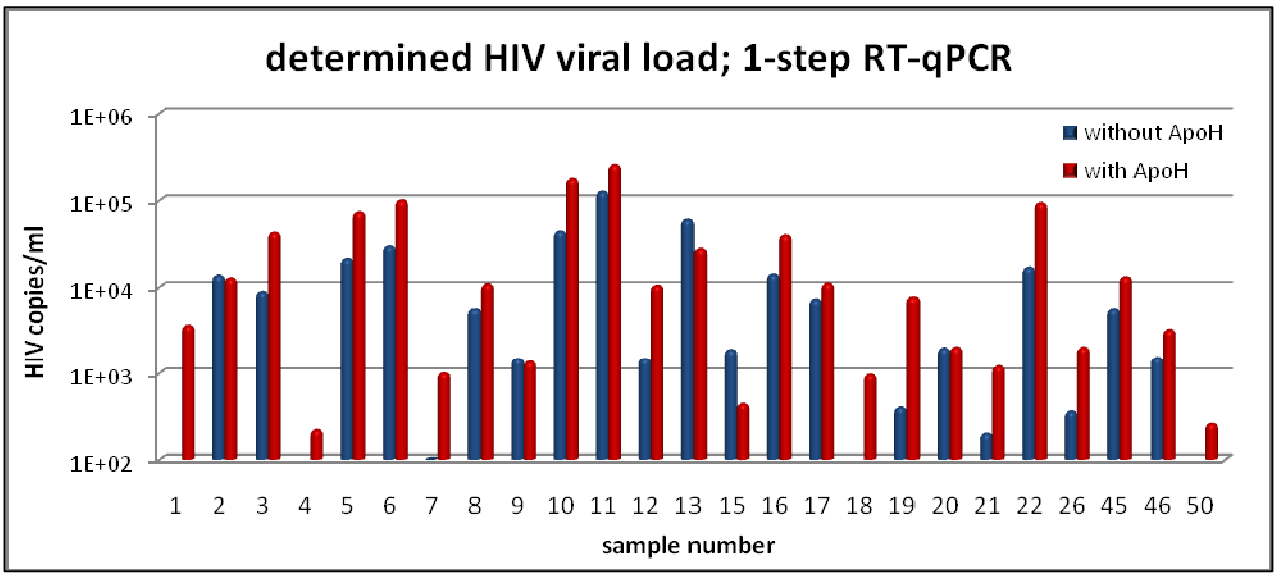


Figure 4: HIV viral load determination with and without pre-treatment with ApoH in clinical samples from Argentinean patients

The ApoH-capture of multiple viruses in clinical samples

All DENV samples from Guyana, Cambodia and St Martin were tested with the Mosquito-born AJ eBiochip array designed to detect a set of pathogens that have in common to be transmitted by mosquitoes. In addition to the expected detection of Dengue, especially in some Guyana samples, plasmodium parasite DNA was detected. These findings could be verified with classical PCR and sequencing identifying *P. vivax*.

The samples from Italy were collected from patients known to have a double infection with HIV and HCV. Most of the patients were under anti-viral treatment, however in some samples both viruses could be detected in RT-qPCR in a single as well as in a multiplex approach (IMC2009040561, IMC2009040562, IMC2009040573, IMC2009040576, IMC2009040578 and IMC2009040579). Mostly the detected signal was enhanced or changed from negative to positive after ApoH-treatment. The samples were also tested positive with the Hepatitis-HIV AJ eBiochip array. (Table 2)

Some of the samples from Argentina were also from HIV/HCV co-infected patients; here we could detect only HCV, most likely because of both, low HIV viremia and not yet completely optimised detection methods. Nevertheless and interestingly, these tools, backed up by ApoH pre-treatment, were able to identify five additional samples as positive for HCV, four of them were also tested positive for HIV. In addition, two samples were tested positive for HBV and HIV, both in single as well as multiplex RT-qPCR. (Table 3)

Table 2. Results for multiplex RT-qPCR and AJ eBiochip array for double positive samples from Italy

			multiplex RT-qPCR				multiplex Hep-HIV eBiochip			
sample information			HCV [Ct-value]		HIV [Ct-value]		HCV		HIV	
IMC number	HIV	HCV	no ApoH	with ApoH	no ApoH	with ApoH	no ApoH	with ApoH	no ApoH	with ApoH
IMC2009040561	HIV+	HCV+	29.3	28.2	31.9	30.1	not tested	not tested	n.d.	positive
IMC2009040562	HIV+	HCV+	30.9	28.5	n.d.	35.4	positive	positive	n.d.	positive
IMC2009040573	HIV+	HCV+	30.8	31.5	n.d.	33.2	positive	positive	n.d.	positive
IMC2009040576	HIV+	HCV+	n.d.	n.d.	37.2	28.6	n.d.	positive	positive	not tested
IMC2009040578	HIV+	HCV+	35.6	26.6	n.d.	26.6	positive	positive	n.d.	positive

n.d.: not detectable

Table 3. Results from multiplex RT-qPCR for double positive samples from Argentina

Multiplex RT-qPCR [copies/rxn]

clinical data			HBV		HCV		HIV	
Coding	HIV	HCV	no ApoH	with ApoH	no ApoH	with ApoH	no ApoH	with ApoH
01	HIV+		145	945	n.d.	n.d.	n.d.	6
09	HIV+		n.d.	8	n.d.	n.d.	n.d.	10
11	HIV+		n.d.	n.d.	115	125	330	795
16	HIV+		n.d.	n.d.	8	16	27	150
26	HIV+		n.d.	n.d.	115	130	12	16
27	HIV+	HCV+	n.d.	n.d.	$1.4x10^3$	$1.8x10^3$	n.d.	n.d.
28	HIV+	HCV+	n.d.	n.d.	$1.7x10^3$	$5.1x10^3$	n.d.	n.d.
42	HIV+		n.d.	n.d.	11	62	n.d.	n.d.
45	HIV+		n.d.	n.d.	455	$1.5x10^3$	55	130
48	HIV+	HCV+	n.d.	n.d.	n.d.	1	n.d.	n.d.
49	HIV+	HCV+	n.d.	n.d.	$1.5x10^3$	700	n.d.	n.d.
50	HIV+	HCV+	n.d.	n.d.	400	$3.5x10^3$	n.d.	5

n.d.: not detectable

EXPECTED IMPACT

Impact in reinforcing competitiveness

Wider applications of nucleic acids detection methods for diagnostic purposes are not only hampered by their complexity but also by their prohibiting costs and all the disadvantages previously enumerated. USDEP approach is complementary to these methods and should permit, through pathogen capture and concentration, better pathogen accessibility to lysis reagents and consequently a better efficiency of nucleic acids extraction and detection. A new potentially lucrative market will be then available. Moreover the possibility that virus concentration by ApoH associated technology could allow pathogen early detection, thus facilitating patients prompt treatment, by increasing the detection threshold of most currently used molecular methods, is expected to facilitate the scientific, technical and commercial expansion not only of the ApoH solid supports but also of molecular diagnostic techniques.
Nucleic acid diagnostics is expected to be the fastest growing area of diagnostics in the years ahead. This market is currently in the hundreds of millions of dollars, but as the need for information about genes in genomics and other research progresses, and **as clinical diagnostic tools** develop, the market for nucleic acid amplification technology will continue to grow. Although PCR is currently the dominant amplification technology used for pathogen detection, USDEP project is emerging to assist this technology and to create new innovative solutions fitting diagnosis field needs. Patents, costs, and ease of use of a technique are forces that drive the development of amplification technology and will shape this dynamic market. ApoH coupled to PCR or Mini-Array technology has to design a basic field original detection kit, that should be easy to use requiring low capabilities and few devices and including a low priced production. The chosen solid support and the ease to implement this technology should help solve these problems.

USDEP has resulted in development of commercially viable capture and concentration tools (ApoH-coated beads and plates) for pre-treatment of infected samples adapted to different diagnostic tools.

USDEP and ApoH competitiveness

The USDEP project has demonstrated that ApoH can be efficiently used as a pre-treatment step to greatly improve the detection threshold and thereby drastically increase the sensitivity for diagnosis of emerging pathogens, regardless of the kind of molecular or immunological techniques used.

There are several huge markets creating opportunities for the ApoH technology. These markets cover different priority fields such as human health, animal health, environment, food safety, plant health sector, etc.

Originally, ApoH-Technologies business model was simple: develop, optimize and sell supports that significantly improve the microbiological diagnosis. Pilot clinical studies using our proprietary technology associated with means of revelation should optimize these supports in relation with laboratories or companies in the concerned area. As a result of the USDEP project a number of ApoH-Technologies products such as ApoH-coated beads and plates have reached the clinical and industrial development state. The sale of our products, licensing, and technology and business partnerships, are the next logical step of the company.

The aim of ApoH-Technologies is to license the specific use of ApoH-coated solid matrices per field or per application, thus proposing a generic capture method to be used for a wide range of pathogens in order to concentrate them from large sample volumes of complex biological mixtures, otherwise poorly amenable for molecular methods. This will result in a more sensitive and thus more secure detection method able to significantly reduce the number of false negative results.

Moreover, some months ago ApoH-Technologies succeeded a fund raising with biotech investors and business angels with the aim to prepare the industrial and business phase development. Both, this fund raising together with the successful clinical proof of concept obtained after the end of the USDEP project, have been reasons to initiate a very active business development through multiple contacts and marketing positions with the food and agro-industry. In our business development strategy, we have targeted this field because it is a very reactive market that has rapidly resulted in industrial partnership contracts for feasibility (validations).

The results obtained during the project will be promoted through the USDEP website, publications in specialized journals, conferences and exhibition presentations, in order to promote products and the technology as widely as possible.

Already contacts have been made with industrial groups well established in the pharmaceutical and diagnostic area. Contracts have been signed with laboratories in the area of food safety. Within the consortium, a business contract was signed with SkuldTech, a USDEP partner, for sensitive detection of Dengue viruses by using Mini-Array kits. Contacts were also established with South American healthcare centres involved in infectious diseases and ApoH-Technologies is keen and active to open a subsidiary company in one of these countries.

Prototype tools developed by SkuldTech and AJ eBiochip are presented below. Clinical validation of these tools has been initiated providing solid proof of concept and providing baseline for further commercial development and introduction of these tools into the market.

SKULDTECH Dengue Mini-Array Kit

A novel diagnosis method able to detect pathogens in one shoot. The Mini-array method established by SKULDTECH, resting on specific PCR and hybridization is an easy to use method dedicated to disseminate in place where well equipped laboratories and well trained technician are not available. This system is up to 10,000 times more sensitive than the Dot Blot method and up to 100 times more sensitive than standard detection of PCR amplified products by electrophoresis. The Mini-Array system allows a rapid and simple qualitative interpretation of results with naked eye.

Ready to use Mini-Array kit available for the 4 Dengue virus serotypes one step detection developed by SKULDTECH. The Dengue Mini-Array diagnostic kit developed through USDEP project is an easy to use method dedicated to disseminate in areas where well equipped laboratories and well trained technicians are not available. It's a low cost and easy to use method which satisfies the need for Dengue rapid screening in resource limited settings.

AJ eBiochip electrochemical microarray

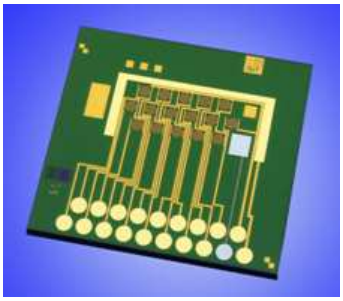
AJ eBIOCHIP is a manufacturer of instruments and test kits based on electrochemical detection on a 16-position microarray chip. The basic instruments offered are eMicroLISA and ePaTOX. For the electrochemical detection, microarrays can either base on ELISA-like protein assays or on hybridization assays for detection of nucleic acids (DNA/RNA).
For point of care analysis a new developed prototype instrument, ePaPROB-Duo, includes the possibility to directly combine microarray detection and PCR in a micro fluidic, disposable cartridge. It can be used for multiple tests on different microorganisms, for example to analyse pathogens, causing tropical fever.

Detection principle:

For the electrochemical detection principle, a silicon biochip serves as the core sensor. The biochip has two parts: a bio-interface and a silicon-based transducer electrode. Each biochip has sixteen sensing positions or microelectrodes. Typical detection of amplified DNA takes less than 8 min in a fully automated assay.
A biochip microarray detecting Dengue Virus, West Nile Virus, Yellow Fiver Virus, Japanese Encephalitis Virus, Chikungunya Virus and Plasmodium sp. is designed and should be tested with clinical samples before implementation into a micro fluidic cartridge.

Typical results from the analysis of products from multiplex PCR on the microarray are shown in. Here, a Dengue sample with approx. 40 copies of cDNA was amplified and clearly positive detected on the microarray. The sensitivity of the method is well comparable to real-time PCR. So far analysis is processed only at the AJ eBiochip laboratory. Samples have to be amplified, giving short nucleic acid fragments (80-100bp), which are afterwards sent to AJ eBiochip.

ePaPROB-Duo



sixteen positon array chip

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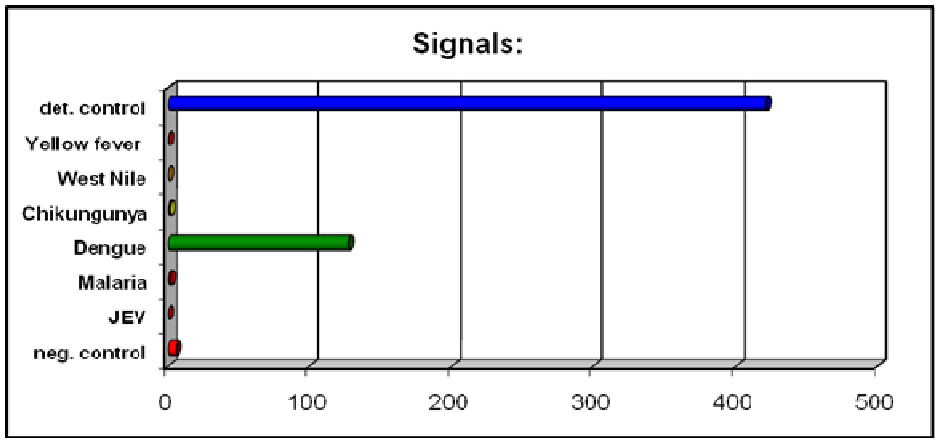

Detection of multiplex PCR products from Dengue cDNA (approx. 40 copies)

Impact on solving societal problems

Infectious diseases are a global concern. Of the annual 15 million deaths attributable to infectious diseases on the planet, 95% occur in the developing world, particularly in the most impoverished areas where individual and general hygiene standards remain very low and prevention policies are non-existent, poorly adapted or insufficiently funded. Moreover, emergence of infections is caused by economic and industrial development (food-borne pathogens, nosocomial and travel-related infections) and by increasing of the ageing population in developed countries such as European. The impact of infectious diseases on economic growth is visible, encompassing both the direct cost of medical care and the reduction in years of healthy life expectancy and productivity because of early death and chronic illnesses. This, in return, leads to a reduction to business and infrastructure investment, social cooperation and social stability. We expect that the use of the products generated from this USDEP project will be paramount for the development of enhanced diagnostic methods and thus contribute to a better diagnosis and prognosis of virus associated infectious diseases.

Impact on innovation aspects

The described technology has a **highly technological and innovative profile**. There are many new and exciting avenues of exploration, each with great potential for biotechnological exploitation. These new directions will take us far beyond the existing state-of-the-art and lead to:

- **conception and development** of biochips usable for easy one-step multiple viruses detection,
- the molecular study of the interaction between ApoH and microorganisms will lead to a **better understanding** of envelop structure of viruses with repercussions in the design of **new drugs** and the improvement of the **vaccines,**
- identification of new pathogen,
- ApoH coupled to various filter supports can be used for the capture and the elimination of pathogen.

Thus, there is a tremendous opportunity, on a global scale, for this technology of biological sample pre-treatment, to provide real benefits in:

- **early diseases diagnosis** and consequently a better individual treatment strategy thus resulting in lower progression over time and less finds spent on long term intensive care,
- in the case of epidemics, ApoH pre-treatment would allow to detect low virus levels allowing a fast application of counter measures necessary to avoid pathogens propagation (**prevention**),
- **therapeutic monitoring** with, consequently, a better adaptation of the therapeutic protocols.
- improvement of existing therapies leading to a better **management** of health policy and **cost reduction** through better diagnosis, individualised treatment and more efficient development pathways for new drugs and therapies and other novel products of the new technologies.
- **capture and identification of pathogenic agents** present in liquids, biological (e.g. transfused blood or transplantation organs) or others (e.g. water) like in the air.

✓ **Impact on networking and business development aspects**

USDEP consortium gathered a large range of skills and capabilities required for innovative diagnostics market launch including technologies capabilities, access to infectious samples, authorities' submission.

Therefore USDEP project leads to:

o Set-up of sound working relationships with European partners which developed complementary expertise able to cover all the required capabilities to develop and to launch new innovative diagnostics for unmet needs.

- Commercial agreement between the members (ApoH & Skuldtech granted licence) for business development purpose.
- SME involvement into negotiation with worldwide major company for partnership on diagnostics developed within the USDEP consortium framework.
- Opportunities to develop some overseas business and trading agreements in function of local needs for microorganism detection.
- Enhancement of SME partners to improve their technologies and their processes in order to go onto the market.

CHALLENGES FACED

USDEP consortium has demonstrated the utility of ApoH-coated supports for capture and concentration of microorganisms capture and concentration, notably for a clear reduction of clinically false negative results. However during the course of this project it has become evident that the efficiency of pathogen detection can be affected by different factors like the molecular detection method chosen (e.g. one-step or two-step qPCR), the nature of sample, etc. Thus, studying in detail these issues for HCV, we have demonstrated that, the enhancement of detection improved by the use of the ApoH capture can be masked when inappropriate primers or RNA dilution are used. The same observations have been made for other pathogens, notably for Dengue. Despite the accomplished improvements by the USDEP consortium, more work is needed to optimize molecular detection techniques in order to standardize and simplify diagnostic procedures.

Collection of relevant clinical samples and obtaining regulatory approvals has been a challenge and a time consuming task USDEP would have needed an additional year to finalize clinical validation as originally planed. Nevertheless, clinical data obtained are sufficient to initiate full commercial validation of ApoH technology.

GENERAL CONCLUSION

The use of ApoH-coated supports allows ultrasensitive pathogen detection that drastically reduces the number of false negative results from clinical diagnostics. Moreover, ApoH technology accelerates and enhances the detection of many kinds of cultivable and non-cultivable bacteria (Gram + and Gram -) from human blood and tissues. Notably, detection of bacterial nosocomial infections has potential to become an important tool to establish appropriate therapeutic management. USDEP has been highly successful in generating several prototypes of diagnostic tools and in providing clinical confirmation of their application.

FUTURE DIRECTIONS & OPEN ENTREPRENEURIAL OPPORTUNITIES

This project has been pivotal to ApoH Technologies business development and to developing collaborative links between several European SMEs and academic centres.
The introduction of an ApoH-capture step can increase the safety of standard diagnostic procedures for the detection of pathogens. The final aim after the USDEP project is to carry out further comprehensive clinical evaluation of the ApoH technology and integrate the ApoH-treatment of samples into diagnostic tests of microorganisms (single or multiple). Following a recent fund raising, ApoH-Technologies has integrated this aim in its development strategies and is already putting efforts to attempt, at least a part, of this ambitious objective. Several industrial partnerships and contracts have recently been initiated with ApoH-technologies in several fields where the microorganism detection is needed and are at the final step of negotiations.

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P06	GenExpress Gesellschaft für Proteindesign mbH	GenExpress	KAISER Marco	Berlin	Germany
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* clinical samples collected in Brazil could not be analysed in the course of the USDEP project since import into the European Union was delayed due to problems with European ethical approval necessary for export from Brazil