



COST ACTION B28
“Array technologies for BSL3 and BSL4 pathogens”

**5th Management Committee and
WG1, WG2, WG3, WG4 and WG5 meetings**

December 10 – 12, 2007
Vienna, Austria

Venue:

Hotel Ibis Wien Messe
Lassallestrasse 7a
A-1020 Vienna
Tel: +43 1 217 700
Fax: +43 1 217 70 556

Local Organiser:

Tanja Kostic
Austrian Research Centers GmbH - ARC
Department of Bioresources
Environmental Molecular Analytics
A-2444 Seibersdorf, Austria
phone: +43 50 550 3635
fax: +43 50 550 3666
email: tanja.kostic@arcs.ac.at



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Invited speaker's program

DNA microarray assays for detection and genotyping of chlamydiae

Konrad Sachse¹, Helmut Hotzel¹, Peter Slickers², Ralf Ehricht²

¹*Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Institute of Molecular Pathogenesis, Jena, Germany,* ²*Clondiag Chip Technologies, Jena, Germany*

The diagnostic test of the future will be expected to provide complex information on the causative agent of an infection beyond the mere identification of the species. As soon as the diagnostician is confronted with this demand for "multi-dimensional" evidence on the pathogenic microorganism the limitations of PCR-based tests become obvious. Detection of subtle differences among strains, such as single-nucleotide polymorphisms and other intra-species variations, is problematic with standard amplification assays, and even in multiplex PCR only a few target regions can be examined in parallel.

DNA microarray technology opens up new possibilities that may be particularly beneficial for laboratory diagnosis of infectious diseases. This highly parallel approach allows any sample DNA to be simultaneously examined using a large number of probes, which may be derived from a polymorphic gene segment and/or from different genomic regions. In essence, this amounts to sequencing the respective genomic site. Thus, DNA microarray-based tests can attain far higher specificity than PCR.

Although microarrays have become a widely accepted tool for mRNA expression monitoring in gene transcription analysis, their use in rapid diagnosis of bacterial and viral pathogens is only emerging. Recent applications include identification and genotyping of mycobacteria, staphylococci, *Escherichia coli*, *Listeria* spp., *Clostridium perfringens*, *Vibrio* spp., as well as neuroinvasive viruses.

In a recent study, we developed a microarray assay for detection and differentiation of *Chlamydia* spp. and *Chlamydophila* spp. [1]. We used the commercially available ArrayTube™ system (Clondiag Chip Technologies, Jena, Germany), which represents a less expensive system for processing low- and high-density DNA arrays. It involves spotted or *in situ* synthesized DNA chips of 3x3 mm size, which are assembled to form the bottom of 1.5-ml plastic micro-reaction tubes. In contrast to other microarray equipment, hybridization and signal processing can be conducted in an easy and rapid fashion on standard laboratory equipment without additional devices, such as hybridisation chambers. Hybridisation signals are amplified by an enzyme-catalysed precipitation reaction. A CCD camera integrated in the ATR-01 reader is used to monitor DNA duplex formation by kinetic measurement of the precipitation reaction at each spot via specific changes in red light transmission.

Hybridisation probes were designed on the basis of a multiple sequence alignment that included 16S rRNA genes, 16S-23S intergenic spacer regions and 23S rRNA genes from 44 chlamydial strains. To identify genomic segments of high discriminatory power, we developed a sequence analysis algorithm designated the "most variable window approach". Using this procedure, species-specific nucleotide polymorphisms in a region of generally high sequence similarity in the 23S rRNA gene were identified. The selected 26-nt probe

sequences were used on two different series of customized microarrays, i.e. combinatorial high-density *in situ* synthesized arrays and low-density spotted arrays. Target DNA was prepared by consensus PCR using one biotinylated primer. Unique species-specific hybridization patterns were obtained for all nine species of the family *Chlamydiaceae* on both microarray types.

After proving the suitability of the present assay for unambiguous species identification of chlamydial cell cultures we used it for direct detection of chlamydiae from clinical tissue [2]. As many clinical samples contain only low numbers of bacteria, sensitivity becomes the crucial parameter in this application. We were able to show that the sensitivity of the microarray assay was equivalent to that of real-time PCR [3], thus rendering it suitable for use in the diagnostic lab.

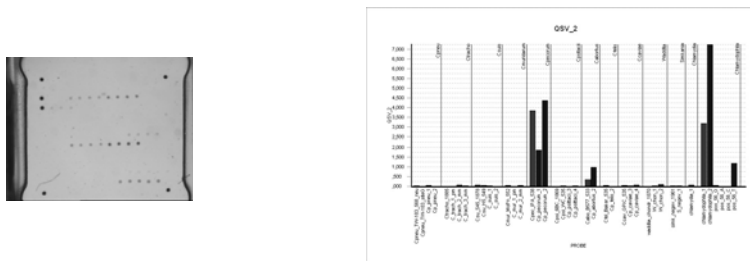


Fig. 1 Identification of *Chlamydophila pecorum* from an ocular swab of an infected calf.

Meanwhile, the above microarray for species differentiation has been complemented with a new array for genotyping of *Chlamydophila psittaci*, the causative agent of psittacosis in birds and humans.

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Parallel Detection and Quantification of Pathogens using DNA Biochips, On-Chip PCR, and Hand-Held Devices

Robert Stedtfeld, Dieter Tournalousse, Farhan Ahmad, Vidya Srinivasan, Tiffany Stedtfeld, Benli Chai, James R. Cole, Erdogan Gulari, James Tiedje, and **Syed A. Hashsham***

**Presenting author:* Associate Professor, Department of Civil and Environmental Engineering and the Center for Microbial Ecology; A126 Research Complex-Engineering, Michigan State University, East Lansing, MI 48824; Phone (517) 355 824; Fax (517) 355 0250; Email: hashsham@egr.msu.edu

With more than 1400 different pathogenic microorganisms having dynamic genomes and extensive allelic variability in their signature genes, it is critical to develop high throughput tools that are able to screen for many pathogens in parallel. There are many desired attributes for such tools including sensitivity, specificity, speed, cost, robustness, sample processing, portability, and the ability to quantify the pathogens. Optimizing all attributes in a single assay is difficult but it is possible to optimize a limited number of parameters for a set of pathogens relevant to a given niche. Using a comprehensive virulence and marker (VMG) gene database encompassing 90 bacterial pathogens and 500 virulence and marker genes, we have developed four different types of parallel detection tools. These are: i) a microfluidic biochip using in situ synthesized 18-mer and 50-mer probes focusing on the specificity, ii) an on-chip PCR array focusing on quantification and enhancing the throughput with respect to the number of samples as well as target pathogens, iii) a high density 50-mer glass slide array using gold and silver nanoparticles focusing on cost, and iv) a microPCR based hand-held device focusing on portability, speed, and sensitivity. On the microfluidic biochip a highly specific assay for 20 waterborne pathogens with little or no false positives was developed. This was accomplished using three to six VMGs per organism and 5 to 20 probes per gene to make the call. To achieve a sensitivity of 0.01 to 0.001%, a multiplex PCR amplification step (combining up to 25 amplicons per reaction mixture) was necessary prior to fluorescent labeling and hybridization. Hybridization signals were highly specific as observed by non-equilibrium dissociation curves obtained for both 18- and 50-mer probes. For the on-chip PCR assay focusing on quantification, approximately 215 genes from 30 pathogens were used to examine the amplification and quantitative capacity of multiple primer sets in 33 nano-liter reaction holes for 12 to 36 samples in parallel. Quantification, sensitivity, and specificity were highly dependent on the genome size and GC content of the targeted organisms and primer characteristics. An empirical equation was developed to predict the copy number of detected amplicons without the use of standard curves. This equation is expected to play a key role in developing parallel screening tools without extensive validation. The third approach focused on reducing the cost of parallel screening by using high density in situ synthesized arrays, labeling with gold and silver nano-particles and scanning using a flatbed scanner (replacing fluorescent dyes and laser scanners). Using this approach, the total cost for equipment and analysis was reduced by 10- to 100-fold without compromising the power of parallel detection and specificity. Automation of gold and silver labeling protocols may further reduce the cost of such assays. The fourth approach focused on developing a

rapid microPCR chip for selected pathogens to be used with portable and point-of-use devices. The PCR chip consisted of microfluidic channels in doped silicon allowing rapid temperature control with minimal power, transparent base and cover for easy visualization with chemiluminescent light source and CCD imaging, and lyophilized PCR reagents. Using these components it is possible to develop less than 2-minute PCR based assays for multiple pathogens. The information gathered during the development of these tools suggests that parallel screening of pathogens using genetic markers has many advantages and should be considered as a method for routine monitoring both under laboratory and field conditions.

We are thankful for support from the National Institutes of Health (1 R01 RR018625), Michigan Economic Development Corporation (GR-476 PO 085P3000517 and 06-1-P1-0557), and MSU Foundation.

Tools for understanding the pathogen in the post genomic era

Guanghai Wu and Muna Anjum

Department of Food and Environmental Safety, Veterinary Laboratories Agency-Weybridge, New Haw, Addlestone, Surrey KT15 3NB, U.K.

The DNA microarray chip offers a new way for biologists to understand the complexities of a pathogen. In our laboratory we have been using microarrays to detect the presence and absence of genes from field and clinical isolates of *Escherichia coli* and *Salmonella*, of both animal and human origins, in comparison to sequenced strains. Information regarding the relationship between different isolates and pathogenetic potential of these isolates have been obtained to understand the evolution of these organisms and develop a better detection method for these pathogens (1, 2, 4, 8)). The microarray platform including array printing, slide processing and procedures for hybridization and data analyzing, using statistical methods, have been well established in our laboratory (7). We have also developed miniturised microarrays using an eppendorf-based system (ArrayTubes) for use in detection of virulence in *E. coli* for pathotyping (3) or antimicrobial resistant genes in Gram negative bacteria, especially *E. coli* and *Salmonella* (6), and in serotyping of somatic *E. coli* antigens (5). Due to the simplicity and rapidness of the ArrayTube systems, they have the potential for development as high-throughput systems in the future and for automation. We are also collaborating and providing technical training to scientists from a number of institutes and universities in the UK and continental Europe in the various array technologies currently being used at the VLA. The virulence and antimicrobial arrays have been commercialized (www.identibac.com.) and are being well received by our users.

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Diversity Arrays Technology (DArT) as a generic tool for microbial diagnostics

Peter Wenzl^{1,a}, Evelyn Hackl^{2,b}, Marianne Konrad-Köszler², Angela Sessitsch², Andrzej Kilian¹

¹Diversity Arrays Technology P/L, 1 Wilf Crane Cr., Yarralumla, Canberra, ACT 2600, Australia (www.DiversityArrays.com); ²Austrian Research Centers GmbH-ARC, 2444 Seibersdorf, Austria;

^apeter@DiversityArrays.com; ^bevelyn.hackl@arcs.ac.at

Diversity Arrays Technology (DArT) is a sequence-independent microarray platform that measures the abundance of DNA fragments in 'representations' prepared from (meta)genomic DNA samples. A representation is a well-defined subset of DNA fragments, generated by restriction-enzyme digestion, adapter ligation and amplification of adapter-ligated fragments (or any other robust and reproducible 'complexity reduction' method). The abundance of fragments in representations is a reflection of single-nucleotide polymorphisms (SNP) at restriction-enzyme sites, insertion-deletion (InDel) polymorphisms between restriction-enzyme sites, or the relative abundance of different species in the case of metagenomic samples.

DArT is established without using any DNA sequence information, yet it produces sequence-ready diagnostic clones. This feature makes DArT particularly attractive for applications that would otherwise require extensive sequencing to capture the genetic diversity of a phylogenetic group. In addition, diagnostic clones are identified and assayed using exactly the same procedure, which obviates the need for a separate assay-development step. To this date, DArT has been applied to more than two dozens of plant species (from mosses to higher plants), several fungal plant pathogens and a few animal species.

We first outline the technology principles, review the most common areas of application in plant science and highlight potential opportunities in the area of microbial diagnostics. We then present the results of a first evaluation of DArT for the purpose of *Salmonella* phage typing. In this project, DArT was adapted to the subtyping of *Salmonella enterica* ssp. *enterica* Enteritidis and Typhimurium serovars as a means to overcome drawbacks associated with traditional, phenotypic phage typing. Phage typing of *S. enterica* is required to precisely identify strains, e.g. in order to trace back sources of infection. We successfully applied DArT-based molecular markers to discriminate among various *S. enterica* phage types by hybridizing representations prepared from individual *S. enterica* strains onto microarrays. By further refining the complexity reduction method used for *Salmonella*, or by using multiple complexity reduction methods, DArT offers a vast potential for identifying significant numbers of phage type-specific markers. The encouraging results of this proof-of-concept study suggest that DArT is a useful, yet underexploited tool for microbial diagnostics.



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Self-Assembled Monolayers for Array Technologies

Erhan Pişkin

Gökhan Demirel, Bora Garipcan, Oğuzhan Bölükbaşı

Self-assembled monolayers (SAMs) are a class of molecular assemblies that are prepared by spontaneous adsorption of molecules from solution onto a solid substrate. Rather than having to use a technique such as chemical vapor deposition or molecular beam epitaxy to add molecules to a surface, self-assembled monolayers can be prepared simply by adding a solution of the desired molecule onto the substrate surface and washing off the excess. A common example is an alkane thiol on gold. Sulfur has particular affinity for gold, and an alkane with a thiol head group will stick to the gold surface and form an ordered assembly with the alkyl chains packing together due to Van der Waals forces. For alkyl thiols on gold, the extended alkyl chains typically orient with an angle of ~ 30 degrees from the perpendicular of the substrate, and are assumed to be in a fully extended linear arrangement. A variety of other self-assembled monolayers can be formed, although there is always debate about the degree to which systems self-assemble. Alkyl thiols are known to assemble on many metals, including silver, copper, palladium, and platinum. Alkyl silane molecules (*e.g.* 3-aminopropyltrimethoxysilane) are another well-known example of self-assembly on silicon oxide surfaces and potentially be of greater technical relevance than alkyl thiol assembly on metals.

Due to their ease of preparation and controllable surface chemical functionality, SAMs represent suitable model systems for studying wetting, corrosion, adhesion, tribology, charge transfer through molecules, and model surfaces for biochemistry and cell biology. Other applications (resistance to etchants and protein adsorption, modified electrodes for electrochemistry) rely on the ability of SAMs to prevent diffusion of other molecules to the surface of the underlying substrate. In the biosensor studies, the process of self-assembled monolayers (SAMs) and more recently, self-assembly of multilayer systems have attracted a great deal of interest. SAMs technology provides a powerful tool for generating monolayers of biological molecules on various solid substrates. The orientation of monolayers offers great versatility in terms of the complex bio-recognition, which might provide a method for the *in vitro* development of biosurfaces that are able to mimic naturally occurring molecular recognition process. In our research group, we are working on the self-assembled mono and multiplayer systems for biosensor applications. Recently, smart self-assembled layers by using poly(N-isopropylacrylamide) were investigated on the silicon surfaces for DNA chip applications. A variety of other self-assembled monolayers are also studied for biosensor application based on the surface plasmon resonance and ellipsometry.

Genomotyping of *Brucella suis*

J. Kieboom, I.A. Voskamp and M.P. Broekhuijsen

TNO Defence, Security and Safety, P.O. Box 45, 2280 AA Rijswijk, The Netherlands

J. Kieboom: jasper.kieboom@tno.nl

Rapid biotyping of potential biological weapons such as *Brucella* is of great importance for protection against these agents. The efficacy of medical countermeasures is partly dependent on such rapid biotyping and identification. By means of molecular biology tools it is possible to identify the *Brucella* species, in some cases even to the biovar level. However, not all known biovars can be identified by means of a single rapid molecular biology method yet. To overcome this problem, whole genome microarray experiments were used to identify new genomic markers, in the genomotyping *Brucella*.

Genomotyping basically uses hybridization of the fluorescently labeled genomic DNA of a strain of interest to the microarray, along with the fluorescently labeled genomic DNA of a reference strain. The latter is typically the strain of which the genome sequence was employed to establish the array. By comparing the ratios of the signal intensities, obtained from the spots on the microarray, one can predict whether a given gene is present or 'divergent' in the strain of interest. Here, 'divergent' refers to either absence of a gene or to a gene that has poor hybridization properties. Thus, the genome of one organism is screened in reference to the genome of a chosen reference organism. A number of studies have investigated genome composition using DNA microarrays. Strain, such as *Francisella tularensis* [1], *Campylobacter jejuni* [2], *Helicobacter pylori* [5], *Staphylococcus aureus* [4], *Vibrio cholerae* [3], and *Streptococcus* [6], have been examined by the DNA microarray genomotyping technique.

The microarray used in our genomotyping study was designed and constructed on the basis of the *Brucella melitensis* 16M and *Brucella suis* 1330 genome sequence. The protocols were optimized and experiments were performed using several *Brucella suis* biovars. Analysis of the microarray experiments revealed some unique oligo-DNA sequences that allowed preliminary biotyping of *Brucella suis*.

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Application of a Non-Cognate Hybridization System (NCHS) microarray for the identification of *Bacillus anthracis* and *Francisella tularensis*

HUYGHE Antoine, FRANCOIS Patrice, BONETTI Eve-Julie, TANGOMO Manuela, CHARBONNIER
Yvan and SCHRENZEL Jacques
*University Hospital of Geneva - Genomic Research Laboratory. University of Geneva, Geneva,
Switzerland*

We report the use of a novel microarray design applied to the characterization of the genetic signature of two pathogenic species: *Bacillus anthracis* and *Francisella tularensis*.

Our strategy, named Non-Cognate Hybridization System (NCHS), is based on 13-mer probes providing all combinations of only two nucleotides, allowing targeting multiple organisms without having prior knowledge of their genomic content. This oligonucleotide length provides an optimal ratio between the number of required NCHS probes and the number of probes actually able to discriminate different targets.

This strategy yielded specific hybridization patterns with either of the two studied pathogens and enabled the characterization of the two *B.anthraxis* toxin-bearing plasmids pXO1 and pXO2. This approach should prove useful in the rapid diagnosis of microorganisms potentially involved in bio-threats.

Identification of causative agents of infectious spondylodiscitis using real-time PCR : advantages and limitations

Léonid M. Irengé¹, Jean-François Durant², Xavier Banse², Michel Lambert², Frédéric Lecouvet²,
and Jean-Luc Gala^{1,2}

¹*Defence Laboratories Department, Belgian Armed Forces, Brussels, Belgium.*

²*Centre of Applied Molecular Technology, Université catholique de Louvain, Brussels, Belgium.*

Infectious spondylodiscitis can be defined as concurrent infection of the intervertebral disc tissue and adjacent vertebrae, observed spontaneously or after invasive spinal procedures or surgery. Hospitals worldwide have witnessed an increase in the prevalence of the disease during the past decade. The main etiologic agents are pyogenic microorganisms, with *Staphylococcus aureus* the most frequently encountered, with the resurgence of tuberculosis owing to the AIDS pandemic, tuberculous spondylodiscitis prevalence is also on the rise.

Magnetic Resonance Imaging (MRI) has improved the diagnosis of infectious spondylodiscitis but cannot discriminate between pyogenic and tuberculous spondylodiscitis. Culture methods of disc aspirate show low sensitivity ($\pm 50\%$).

We have published a work on contribution of molecular methods in identification of etiologic agents of infectious spondylodiscitis, but these methods have drawbacks precluding them of being used in routine diagnosis : carryover contamination, lack of automation and difficulty of standardization between laboratories, cost, need of highly trained personnel. Sequencing is time consuming and takes at least 24 hours for definitive identification of the majority of species. This report is a first step towards addressing those questions.

17 patients with clinical and magnetic resonance imaging (MRI) findings of spondylodiscitis were included in the study. Conventional culture and molecular identification methods were performed on the same puncture disc samples. DNA was extracted using manual NucliSens® miniMag magnetic extraction (Biomérieux Inc., Boxtel, The Netherlands) and processed with molecular methods. Molecular methods consisted of three duplex real-time PCR assays. The first assay is a broad-range duplex real-time PCR targeting 16S rRNA gene for amplification of all bacteria and discrimination of Gram-positive with Gram-negative bacteria (PCR 16S G+/G-). A second duplex real-time PCR amplifies targets specific for *Staphylococcus aureus* (*purA* gene) and methicillin Resistance genetic (*mecA*) determinant respectively and allows discrimination of Methicillin-Resistant *Staphylococcus aureus* (MRSA) with Methicillin-Sensitive *Staphylococcus aureus* (MSSA). For other non-*S.aureus* Gram-positive and Gram-negative samples, definitive identification was achieved by comparing the sequence of the amplicon with public available sequence databases.

A third duplex real-time targeting *Mycobacterium tuberculosis complex* (MTC) specific insertion sequence IS6110 and the common mycobacteria heat shock protein (*hsp65*) gene was designed and performed on

samples of patients suspected of tuberculous spondylodiscitis. Further species identification of non-MTC mycobacteria was also achieved by sequence comparison of hsp65 amplicons with public available sequence databases. Molecular methods for identification of Gram-positive and Gram-negative bacteria showed a higher sensitivity and accuracy compared with conventional microbiologic identification methods (100% vs 62.5% and 100% vs 85% respectively) but the overall performance of the two methods were not significantly different ($p > 0.05$ with the McNemar χ^2 statistic test). Molecular method for identification of Mycobacteria had a higher sensitivity and accuracy (100% vs 55.6% and 100% vs 81% respectively) and showed a significant better overall performance ($p < 0.05$ with the McNemar χ^2 statistic test). DNA-based methods are highly sensitive and specific and are able to deal effectively with the difficulty of identification of microorganisms responsible of spondylodiscitis, especially tuberculous mycobacteria. These results show also that the prevalence of spondylodiscitis caused by non-MTC mycobacteria is probably underestimated as its etiologic agents are difficult to culture. Accordingly, molecular methods can be of paramount utility in their identification. However, in order to be implemented in routine laboratories, real-time PCR must be combined with a more rapid identification, notably micro-arrays.

Diagnosis of *Francisella tularensis* in organs of a naturally infected common squirrel monkey (*Saimiri sciureus*) and zoonotic transmission to a veterinarian

Joachim Frey, Carlos Abril and Paola Pilo

Institute of Veterinary Bacteriology, Universität Bern, Switzerland

Francisella tularensis, a small Gram-negative facultative intracellular bacterium, is the causative agent of tularaemia. The disease, which is transmitted mostly by vectors such as ticks, flies and mosquitoes, is endemic in many parts of the northern hemisphere. Among animals, the most affected species belong to rodents and lagomorphs, in particular hares. However, in the recent years, many cases of tularaemia among small monkeys in zoos were reported. We have developed a real-time PCR that allows quantifying *F. tularensis* in tissue samples. Using this method, we identified the spleen and the kidney as the most heavily infected organ containing up to 400 *F. tularensis* bacteria per simian host cell in two common squirrel monkeys (*Saimiri sciureus*) from a zoo that died of tularaemia. In other organs such as the brain, *F. tularensis* was detected at much lower titres. The strain that caused the infection was identified as *F. tularensis* subsp. *holarctica* biovar I, which is susceptible to erythromycin. The high number of *F. tularensis* present in soft organs such as spleen, liver and kidney represents a high risk for persons handling such carcasses and explains the transmission of the disease to a pathologist during post-mortem analysis. Herein, we show that real-time PCR allows a reliable and rapid diagnosis of *F. tularensis* directly from tissue samples of infected animals, which is crucial in order to attempt accurate prophylactic measures, especially in cases where humans or other animals have been exposed to this highly contagious pathogen.

ARC microbial diagnostic microarrays for environmental and food analysis

L. Bodrossy¹, T. Kostić¹, B. Sandjong-Tankouo¹, N. Stralis-Pavese¹, P. Wenzl², E. Hackl¹, A. Kilian²
and **A. Sessitsch**¹

¹*Austrian Research Centers GmbH (ARC), Dept. of Bioresources, A-2444 Seibersdorf, Austria*

²*DArT Pty Ltd., POB7141, Yarralumla, ACT2600, Australia*

(angela.sessitsch@arcs.ac.at)

Microbial diagnostic microarrays are molecular tools used for simultaneous identification of microorganisms in food, clinical and environmental samples. The main advantages of MDMs are high throughput, parallelism and miniaturization of the detection system. Furthermore, both high specificity and high sensitivity of the detection can be achieved. Different microarray systems including microarrays based on short or long oligonucleotide probes, sequence-specific end-labelling of probes (SSELO) or approaches based on whole genome differences have been applied in our laboratory. These different approaches exhibit certain advantages and limitations and are suitable for different applications. In addition, depending in the application, various marker genes have been used for probe design including pathogenicity-related markers, housekeeping genes as well as key genes involved in environmental processes. In some cases, whole genome approaches (Diversity Arrays) have been applied as no suitable marker genes to be used for probe design could be identified.

Microbial diagnostic microarrays developed at our institute target environmental bacteria as well as human pathogens. All known (either by cultivation or by cultivation-independent analysis) methane oxidizing bacteria can be easily identified by a microarray, which is based on sequence differences within the *pmoA* gene encoding the key enzyme methane monooxygenase. In the field of human pathogen detection, one of our developments is able to detect and identify common waterborne pathogens and indicator organisms. This microarray is based on the combination of a unique labeling method (SSELO), a novel concept of competitive oligonucleotides and the *gyrB* gene as phylogenetic marker resulting in high specificity and sensitivity. Other ARC microarrays have been developed for the typing of food pathogens rather than for their detection. The ARC Salmonella Serotyping Array is able to distinguish more than 40 *S. enterica* serotypes prevalent in Europe and has the potential to replace classical serotyping. It is based on specific short oligonucleotides, which target two housekeeping genes (*gyrB*, *atpD*) and two flagellin genes (*fliC*, *fljB*). The development of additional microarray-based typing methods is on-going.

Quantitative multiplex detection of plant pathogens using PRI-lock probes and universal, ultra-high-throughput real-time PCR on OpenArrays™

R. van Doorn, M. Szemes, P. Bonants, **C.D. Schoen**

Plant Research International B.V., The Netherlands

cor.schoen@wur.nl

Current technologies for multiplex, quantitative analyses frequently suffer from compromises between the level of multiplexing, throughput and accuracy of quantification. In general, for the detection of nucleic acids, microarrays provide very high level of multiplexing, but less accurate quantification and usually low throughput. At present, real-time, quantitative PCR provides the most reliable means of target quantification, and it is suitable for the analysis of a relatively high number of samples. The achievable level of multiplexing, however, is low.

Nano-scale technology, provides high-density and low-volume microchambers, which could accommodate very high number of reactions, performed under standard conditions. Many of these systems are still at the experimental phase, and are not capable of monitoring the fluorescent signals in real time for each microwell, which is required for quantification.

Recently, a conceptually new, ultra-high-throughput platform has become available for real-time PCR, capable of accommodating more than 3000 reactions per array. The OpenArray™-s have 48 subarrays, allowing parallel testing of up to 48 samples, and each subarray contains 64 microscopic through-holes of 33 nL volume. The primers are pre-loaded into the holes, while the sample along with the reagents are auto-loaded due to surface tension, provided by the hydrophilic coating of the holes and the hydrophobic surface of the array.

Plant Research International recently has developed PRI-Lock probes for multiplex detection which provide flexibility, and bridge the gap between target-specific recognition and high-throughput amplification using universal but unique primer pairs and a generic TaqMan probe. PRI-lock probes are long oligonucleotides, similar in structure to padlock probes. They contain artificially selected primer sites and a TaqMan probe region, flanked by target complementary regions.

In this study, we have characterized the quantitation power of circularizable ligation probes, and report the development of a high-throughput, quantitative multiplex diagnostic assay based on the described principle.

DNA Microarray Technique for Detection and Identification of Viruses Causing Encephalitis and Hemorrhagic Fever

Henrik Nordström^{1,2}, Kerstin I. Falk^{1,2}, Annelie Waldén³, Peter Nilsson³ and Åke Lundkvist^{1,2}

¹Swedish Institute for Infectious Disease Control, SE-17182 Solna, Sweden.

²Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, SE-17177 Stockholm, Sweden.

³Royal Institute of Technology, Department of Biotechnology, SE-10044 Stockholm, Sweden.

We are developing DNA microarray methods for viruses causing encephalitis and hemorrhagic fever for which rapid and correct virus identification is important making broad screening methods useful. Microarrays have been designed for Hantaviruses, Flaviviruses and a group of hemorrhagic fever viruses. Highly degenerated primers were used for amplification of sample material before hybridization to the Hantavirus and Flavivirus microarrays while random amplification was tested on the group of hemorrhagic fever viruses. The results are promising and tests on clinical Dengue samples demonstrate the potential and usefulness of virus identification methods based on microarray technique.

Progress towards Use of Microarrays as a Diagnostic Tool; Lights at the end of the Tunnel?

Karen Kempell, Sonal Shah, Susanna Sherwin, Richard Vipond, Nigel Silman

HPA Diagnostic Technologies, Department of Novel and Dangerous Pathogens, HPA Centre for Emergency Preparedness and Response, Porton Down, Salisbury, Wiltshire SP4 0JG.

Many groups including our own are developing pan-pathogen microarrays for use in detection of pathogens in clinical and environmental samples. The pan-pathogen array developed 'in house', contains approximately 2000 oligonucleotide probes for around 130 different bacterial and viral pathogens, mainly bioterrorism-associated pathogens. As part of this larger array, we have also designed a subset of probes for use in detection of meningitis-associated bacterial pathogens e.g. *Neisseria meningitidis* and *Streptococcus pneumoniae* and for molecular serotyping different strains of *N. meningitidis*. We have tested this array with purified nucleic acids from a number of bacterial and viral pathogens and are utilising these data to select oligonucleotide probes of high-specificity for future focused array development. We are also currently validating this complex array for use in detection of pathogens in clinical samples and have been investigating methods to optimize the assay with a view to future implementation for routine use.

A number of technical issues have been uncovered during the course of these analyses, (1) technical variation due to inconsistencies in local hybridization conditions (2) probe background hybridisation signals due to non-specific target binding (3) technical issues due to operator variation (4) technical variation in clinical samples due to contaminating nucleic acids, e.g. clinical samples and tissue culture grown viruses. Here we present more recent data outlining (1) improvements in our hybridisation methods (2) validation of our array for a number of pathogens & selection of highly-specific pathogen probes (3) conclusions from our multi-operator microarray quality assessment (4) methods to discern pathogen specific signals in clinical material. We suggest strategies to minimise technical variations and recommendations for improved general diagnostic microarray assay methodologies.

The use of Multiple Locus VNTR Analysis (MLVA) for the typing of *Brucella*

K. Walravens¹, F. Grégoire², J. Godfroid³, A. Linden² and C. Saegerman²

¹*CODA-CERVA, Brussels, Belgium*

²*University of Liege, Liege, Belgium*

³*University of Pretoria, Pretoria, South Africa.*

The genus *Brucella* is currently divided into 8 species, subdivided into different biovars. The classification of the genus into species is mainly based on differences in host preference and virulence properties, whereas the classification into biovars refers mainly to epidemiological considerations.. Important resources are needed to maintain and perform the whole battery of tests. Moreover, some of these assays are poorly standardized. The development of molecular techniques for the typing of *Brucella* has been hampered by the great genomic homogeneity between the different *Brucella* species. However, the completion of genome sequencing projects allowed the identification of Variable Number Tandem repeats (VNTR) that have been subsequently used to develop and validate MLVA typing. The analysis of a subset of 15 selected loci allows a robust discrimination between terrestrial species (Leflèche *et al.*, 2006, BMC Microbiol. 6:9) and classifies *Brucella* strains isolated from marine mammals into two distinct species (*B. ceti* and *B. pinnipedialis*). The evaluation of MLVA for the molecular typing of *Brucella* with the aim to perform epidemiological investigations is under evaluation. However, the completion of genome sequencing projects allowed the identification of Variable Number Tandem Repeats (VNTR) that have been used to develop and validate a MLVA typing method. The analysis of a subset of 15 selected loci allows a robust discrimination between terrestrial species (Leflèche *et al.*, 2006, BMC Microbiol. 6:9) and classifies *Brucella* strains isolated from marine mammals into two distinct species (*B. ceti* and *B. pinnipedialis*). The evaluation of MLVA for the molecular typing of *Brucella* with the aim to perform epidemiological investigations is under evaluation. Analysis of the discriminatory power and stability of different sets of loci for the typing of *Brucella abortus* and *Brucella suis* biovar 2 isolated in Belgium is under way and results will be discussed.

Genetic characteristics of new HBV strains in Sub-Saharan Africa and SE-Asia: new subtypes, unclassifiable strains and multiple double and triple recombinations

CP. Muller¹, CM. Olinger¹, M. Njayou², V Venard³, AOB Oyefolu⁴, JJ Muyembe Tamfum⁵, YK Nèbie⁶,
I. Maïga⁷, SA Omilabu⁴, A. Le Faou⁸

¹*Institute of Immunology, National Public Health Laboratory, Luxembourg*

²*Centre de Biotechnologie–Nkolbisson, Yaoundé, Cameroon*

³*Unité Mixte de Recherche 7565 UHP-CNRS, Faculté de Médecine, Nancy, France*

⁴*College of Medicine of the University of Lagos, Nigeria*

⁵*National Institute of Biomedical Research, Kinshasa, Democratic Republic of Congo*

⁶*Biomedical Research Center Muraz, Bobo-Dioulasso*

⁷*Hôpital du Point G, Bamako, Mali*

⁸*Institut Pasteur, Bangui, Central African Republic*

Sub-Saharan Africa suffers from an excessively high endemicity of hepatitis B virus (HBV), but until recently little was known about the prevalent genotypes. In this study, we investigated the preS1/preS2/S genes of >200 viruses, >150 preCore/Core gene sequences and representative complete genomes collected from 15 locations in Mali, Burkina Faso, Togo, Benin, Nigeria, Cameroon, Democratic Republic of Congo and Central African Republic. Except for Cameroon (18/22 genotype A), >85% of sequences from each location belonged to genotype E with a very low diversity (1.67%) throughout West and Central Africa. In contrast genotype A strains were highly diverse (5.1 %) and separated into three subtypes including two new ones (A4, A5. The low diversity suggests that HBV/E may have a short evolutionary history. It would take only 200 years for the strain diversity of HBV/E viruses to develop from a single introductory event suggesting a short evolutionary history and explaining its conspicuous absence in the New World, despite the forced immigration of slaves from West Africa, until the early nineteenth century. Infection during infancy is mostly associated with chronic carrier status but could hardly account for the explosive spread of virtually identical viruses in Africa. In SE-Asia, detailed phylogenetic analysis of strains from Laos revealed multiple different subtypes of B and C, mixed infections as well as numerous related new strains that are non-classifiable. Both in Africa and Asia a high frequency (>20%) of mixed infections were found and many recombinations between the new non-classifiable. In Nigeria a triple recombination of genotype E/D and A was found.

Spread and evolution of HPAI H5N1 in poultry, humans and wild-birds in Sub-Saharan Africa

Muller CP¹, Forbi JC², Mbah PO³, Olinger CM⁴, Tarnagda Z⁵, Tahita MC⁵, Sow A⁶, Osterhaus ADME⁷, Fouchier RA⁷, Ouedraogo JB⁸, Owoade AA⁹

¹*Ducatez MF, Institute of Immunology, Luxembourg*

²*Institute of Immunology, Luxembourg; Innovative Biotech, Abuja/Keffi, Nigeria*

³*Institute of Immunology, Luxembourg; Catholic Medical Center, Bamenda, Cameroon*

⁴*Institute of Immunology, Luxembourg*

⁵*Institut de Recherche en Santé, Bobo-Dioulasso, Burkina Faso*

⁶*Laboratoire National de l'Elevage, Ouagadougou, Burkina Faso*

⁷*Erasmus Medical Center, Rotterdam, Netherlands*

⁸*Institut de Recherche en Santé, Bobo-Dioulasso, Burkina Faso*

⁹*University of Ibadan, Nigeria*

Phylogeny and substitution rates of H5N1 strains from Nigeria, the first African country to report H5N1, identified three sublineages in February 2006 which were probably independently introduced. These three sublineages have antigenic differences and a distinct geographic distribution: sublineage A (initially Southwest Nigeria, Lagos, Niger), B (initially Southwest Nigeria, Lagos, Egypt, Djibouti) and C (initially Northern Nigeria, Burkina Faso, Sudan, Ivory Coast) within Africa and in the meantime beyond. Evidence of several reassortments between these sublineages is currently emerging in several W-African countries. In 2006 all reported human cases in Africa (Egypt, Djibouti) were caused by sublineage B. In January 2007 the first human case was reported from Lagos after contact with poultry from a life-bird market. Also serological data from poultry workers in Nigeria, Burkina Faso and Cameroon, where H5N1 was clinically suspected in poultry (even prior to Nigeria) will be presented. In Burkina Faso 48 hooded vultures (lineage C) were found sick or dead as a result of spill-back of H5N1 from poultry to wild birds. Vultures are both vectors and conspicuous sentinels, similar to swans in Europe. In Sub-Saharan Africa the virus has killed at least one human and continues to spread to new countries such as Ghana. The Luxembourg AI Response team has set up diagnostic laboratories and has trained staff in or from Nigeria, Niger, Burkina Faso, Ruanda, Egypt, Cameroon, and Kyrgyzstan. Learning from the experience in implementing molecular surveillance techniques under biosafety condition in resource poor countries is critical for world-wide HPAI H5N1 surveillance.

Synthetic Mimicry of the CD4-Binding Site of HIV-1 gp120 for the Design of Immunogens

Raimo Franke¹, Tatjana Hirsch¹, Sascha Antoni², Ursula Dietrich² and **Jutta Eichler**¹

¹*Heimholtz Centre for Infection Research, Braunschweig, Germany*

²*Georg-Speyer-Haus, Institute for Biomedical Research, Frankfurt, Germany*

Entry of the virus HIV-1 into its host cells is initiated by specific interaction of the HIV-1 exterior envelope glycoprotein gp120 with the receptor CD4 on T-lymphocytes and other CD4-expressing cells. The binding site of gp120 for CD4 (CD4bs) represents a conserved region in this otherwise highly variable protein. Furthermore, the epitope of the broadly neutralizing anti-HIV-1 antibody mAb b12 has been found to overlap the CD4bs. Synthetic mimetics of this binding site are therefore promising immunogen candidates for the elicitation of virus-neutralizing antibodies.

Based on the x-ray crystal structure of core gp120 in complex with two extracellular CD4 domains [1], we have designed and generated a range of scaffolded peptides [2], which present the fragments making up the discontinuous CD4bs (i.e. 365-373, 424-433 and 454-460). Some of these peptides were found to compete with gp120 for binding to CD4 and mAb b12, respectively [3].

Polyclonal antisera raised against a CD4bs mimetic peptide were found to recognize gp120 with a specificity related to that of mAb b12 [4]. Furthermore, affinity-purified antibodies from these antisera recognized gp120 in its native trimeric conformation, as well as complete, membrane bound trimeric HIV envelope protein. Ongoing studies addressing the issue of structural mimicry of the CD4bs of gp120 synthetic peptides, will guide the design of improved synthetic mimetics of the CD4 binding site of gp120 immunogen candidates for the elicitation of broadly neutralising anti-HIV-1 antibodies.

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Incidence of BSL 2 and BSL 3 pathogens in ticks from Luxembourg

Anna L Reye, Judith Huebschen, **Claude P Muller**

Institute of Immunology, National Public Health Laboratory, L-1950 Luxembourg

In Europe several tick-borne pathogens classified as BSL 2 or BSL 3 lead to serious infections in humans. Although the interest in incidence data these pathogens increases steadily, currently only incomplete data are available for Europe and none for Luxembourg. This project aims to determine the prevalence of nine tick-borne pathogen genera in Luxembourg, adding another piece to the jigsaw of their geographical distribution in Europe. Six of the investigated genera are classified as BSL 2 (*Anaplasma* sp., *Bartonella* sp., *Borrelia burgdorferi* s.l., *Ehrlichia* sp., *Rickettsia* sp., *Babesia* sp.). The human pathogenic species of the other 3 genera are classified as BSL 3 (*Coxiella* sp., *Francisella* sp., TBE virus). Ticks from more than 30 sites throughout Luxembourg have been collected and morphologically identified. The different genera will be analysed by PCR using specific primers. Species identification will then be performed by sequencing.

The most commonly reported tick-borne infection is Lyme borreliosis, caused by *Borrelia burgdorferi* sensu lato. It is a multi-system disorder which can affect the skin, heart, nervous system, and to a lesser extent the eyes, kidneys, and liver. *B. burgdorferi* s.l. is a gram-negative bacterium belonging to the family of Spirochaetaceae. Currently 11 *Borrelia* species are known, of which at least three (*B. afzelii*, *B. burgdorferi* sensu stricto, *B. garinii*) are pathogenic in humans. The bacteria are transmitted during the blood feeding of ticks of the *Ixodes* genus; main vectors in Europe are *I. ricinus* and *I. persulcatus*. In most cases the infection is acquired from reservoir hosts by ticks at larval or nymphal stages and then transmitted horizontally by nymphs and adults. The majority of human cases reported from Western Europe can be traced back to the feeding of nymphs.

Proteomic analysis of bacteria from the *Rickettsia* genus.

Patricia Renesto and Didier Raoult

Unité des Rickettsies - CNRS UMR 6020, Marseille, France

In the last decade, we have experienced a major revolution in the biological sciences resulting from a tremendous flux of genome sequence informations. Availability of complete microbial genome sequences has greatly benefited our knowledge of micro-organisms with regard to their evolutionary history, the metabolic processes they catalyze, as well as their antigenic proteins and virulence factors. The availability of genome sequences of bacteria from the genus *Rickettsia* was a starting point for a comprehensive proteome analysis of these obligate intracellular pathogens. Here, we will review insights gained from the proteomic analysis of rickettsiae. Obtained data included the establishment of the proteome maps of *R. conorii* and *R. prowazekii* by two-dimensional electrophoresis coupled with high-throughput matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Such a classical proteome analysis was complemented by capillary chromatography/mass spectrometry combinations which allowed characterization of 165 proteins out of 1,512 predicted protein-coding genes of *R. felis*. Another relevant application was immunoproteomics. Thus, in the case of *R. conorii* the antigenic properties of GroEL were demonstrated. In addition, overlay assays on *R. conorii* and *R. prowazekii* 2D-gels were shown to be very convenient for the identification of putative rickettsial adhesins, namely the α -peptide and the paralogous proteins encoded by RC1281 and RC1282. The comparative analysis of the avirulent (Madrid E) vs a virulent strain of *R. prowazekii* (RP22) by the DIGE approach, revealed a difference in the expression of rickettsial adhesins between both strains. Because of the intrinsic difficulty in working with these intracellular bacteria and the lack of adequate methods for their genetic manipulation, the molecular mechanisms involved rickettsial pathogenicity is still poorly investigated. Our work evidenced that the proteomic approach is a valuable tool for identifying protein candidates of value for the development of diagnosis, therapeutics and vaccines against rickettsial diseases.

The Real-Time immuno-PCR: an ultrasensitive detection tool.

Zorzi Willy and ElMoualij Benaissa

Center of research on Prion Proteins, University of Liège, 4000, Belgium

The immuno-Polymerase Chain Reaction¹⁻² (immuno-PCR) is a technology coupling an antibody detection step, similar to an Enzyme-Linked ImmunoSorbent Assay (ELISA), with amplification of a DNA probe linked to the detection antibody by PCR. This method combines therefore the specificity of antibody-antigen recognition and the sensitivity of exponential nucleic acid amplification by PCR. Because of the efficiency of nucleic acid amplification, immuno-PCR typically leads to a 10- to 10,000-fold increase in sensitivity compared to an analogous ELISA³.

This short review summarizes a list of biological applications using Real Time immuno-PCR (iPCR) and showing that this technology could be applied on clinical (blood, urine, CSF,...), environmental (water, biofilm, sludge,...) or agro-food (milk, salad, ...) matrices to detect protein⁴⁻⁶, hormone, toxin⁷, virus⁸ and bacteria⁹.

Furthermore, a Strep-EC project named "Neuroscreen" (LSHB-CT-2006-037719) will be introduced to illustrate a specific immuno-PCR application to the differential diagnostic of Alzheimer, Parkinson and Creutzfeldt-Jakob diseases. This diagnostic will be achieved by the detection of specific direct and indirect amyloid-related markers in the cerebrospinal fluid and/or in blood, with new derivative products of nano/micro particles biosciences.

This work is supported by the STREP "Neuroscreen" (LSHB-CT-2006-037719), the "Région Wallonne" (R.Rwal. 415754 - i-Detect ; R.Rwal. 0845 - Protéinopathies) and the "Fond Social Européen (R.EURO 0557).

Contact: willy.zorzi@ulg.ac.be

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Recent advances in the structural and functional elucidations of lipopolysaccharide of *Coxiella burnetii*

Rudolf Toman, Pavol Vadovic, Ludovit Skultety, Katarina Palkovicova, Robert Ihnatko,
Eva Betinova, Andrea Fuleova

Laboratory for Diagnosis and Prevention of Rickettsial and Chlamydial Infections, Institute of Virology, Slovak Academy of Sciences, 845 05 Bratislava, Slovak Republic

Coxiella burnetii, the etiological agent of Q fever, is found worldwide and is responsible for an acute and potentially severe disease characterized by pneumonitis, hepatitis, and a significant incidence of neurologic complications. Following infection, the microbe has been observed to persist in human and animal tissues for long periods. Persistent infections in humans may lead to chronic disease in the form of endocarditis which is often fatal. A lipopolysaccharide (LPS) has been considered to be a major determinant of virulence expression and infection of *C. burnetii*. It is a predominant surface antigen, capable of inducing antibody response, and it is considered to be a protective immunogen. Because of its important biological properties, the structure/function relationship studies are of potential interest. The LPS contains a noticeable amount of two sugars virenose (Vir) and dihydrohydroxystreptose (Strep), which have not been found in other LPSs and can be considered as important biomarkers of the bacterium. Both sugars are located in the O-polysaccharide chain of LPS, mostly in terminal positions. In the later stages of infection, a remarkable decrease in the serological activity of LPS was observed when these sugars were selectively released. This indicates that most so-called phase I antibodies are directed against the epitopes containing Vir and Strep. The O-specific chain containing Vir and Strep is involved in the LPS signaling through the TLR4/MD2 complex in contrast with the truncated (R) LPS where the signaling proceeded through CR3. The structure of *C. burnetii* lipid A differs from those found for strongly endotoxic enterobacterial lipid A. This could be the reasons for the observed weak biological activities of the LPS against host immune cells, thereby contributing to the persistent inflammatory reactions during infection.

Hypotetical lipoprotein FTT1103 - a new potential virulence factor of *Francisella tularensis*?

Adela Straskova

CAS FMHS, University of Defence, Czech Republic

Francisella tularensis is characterized as Gram-negative, facultative intracellular, non-motile coccobacillus, the causative agent of severe disease-tularemia. It is classified as a category A agent, i.e. biological weapon, because of its high infectivity (already a 10 cfu can cause a disease), unavailability of efficient vaccine and easy way of dissemination (primarily aerosol). The virulence factors of *F.tularensis* are still quite unknown. Until recently the understanding of the basis of *Francisella* pathogenicity was hindered by the lack of genetic tools. Previously studies of potential virulence factors were considered on surface determinants such as lipopolysaccharide (LPS) with his unique O-sidechain, the capsule or the 17kDa membrane protein Tul4. With the improvement in genetic branch we know one of the most important parts of *Francisella* genom which is responsible for intracellular surviving of these bacteria in host cells—the *Francisella* pathogenicity island (FPI).

Our strategy is based on comparative proteome studies of fractions enriched for membrane-associated proteins from *Francisella tularensis* subsp.*tularensis* (type A) and *F.tularensis* subsp. *holarctica* (type B) strains. In this study were identified proteins that exhibit differential expression comparing less and fully virulent *Francisella tularensis* strains. Hence, these proteins can represent candidates for novel virulence factors of *Francisella tularensis*. We have chosen three of these candidates and constructed selective deletion mutants for genes encoding these proteins. Based on bioinformatic searches all these proteins were classified as lipoproteins. Lipoprotein FTT1103 showed homology with DsbA family and lipoprotein FTT1591 was classified as member of the VacJ family. The last one, FTT1182, was classified by genomic analyzes and also it is the member of the VacJ family. All these lipoproteins are also known as an important virulence factors in other bacteria. Testing of their virulence potential in *Francisella tularensis* is currently underway.

Proteomic analysis of lipid rafts of mouse monocyte-macrophage cell line J774 during *Francisella tularensis* LVS infection

Anetta Hartlova

CAS FMHS, University of Defence, Czech Republic

There is a strong evidence that plasma membrane is not homogeneous but distinctly organized into domains containing a selective set of molecules involved in signal transduction, membrane trafficking and cell-cell interaction. It has been demonstrated that various pathogens target the domains (also called lipid rafts) to enter the host cells as an infectious strategy. The aim of our study was to examine whether the raft-associated proteins play role in the interaction of mouse monocyte-macrophage cell line J774.2 with the intracellular bacteria *Francisella tularensis* LVS. At first we isolated the membrane domains from noninfectious J774.2 cells and followed by characterization by several proteomic tools. To unravel the molecular mechanism of *Francisella tularensis* infection we have to undertake another analysis of different expressed proteins in the lipid rafts of noninfectious and infectious cells.

Discovery of new therapeutic targets for infectious diseases by the informational spectrum method

Nevena Veljkovic, Sanja Glisic, **Veljko Veljkovic**

Center for Multidisciplinary Research, Institute of Nuclear Sciences VINCA,

P.O.Box 522, 11001 Belgrade, Serbia

The field of bioinformatics has become a major part of the drug discovery pipeline playing a key role in improvement and acceleration of this time and money consuming process. Here we review the application of the informational spectrum method, a virtual spectroscopy method for structure/function analysis of proteins, in identification of functional protein domains representing candidate therapeutic targets for drugs against HIV-1, anthrax, highly pathogenic influenza virus H5N1 and adenovirus type 14.

Multiplexing the detection of Biothreat Agents

Ingmar Janse

Dutch Institute for Public Health and the Environment (RIVM)

There is a need for rapid and reliable diagnostic tools for the detection of agents that can have a major public health impact. Identification and treatment of exposed individuals will be critical to their survival and to reduce the spread of disease. Molecular detection methods can achieve the required speed and specificity, moreover, these methods can be easily scaled-up which is important for speeding up investigations and control of disease progression in (natural) outbreak situations. An essential prerequisite for molecular detection methods is the ability to simultaneously detect multiple genetic markers. Co-amplification of control samples reduces false-negatives and the inspection of multiple genetic targets enhances the reliability of detecting an organism (reducing false-positives) and enables the parallel detection of multiple pathogens.

For the detection of a number of highly pathogenic bacteria we have developed several 4-plex real-time PCR assays detecting 3 diagnostic targets per organism and 1 control target. These assays have been thoroughly validated and are being incorporated in our screening protocols. These assays now include *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis* as these species are considered to pose the greatest risk to public safety due to their potential to cause high rates of morbidity and mortality in humans. Soon we will also include pathogens from the genera *Brucella*, *Coxiella* and *Burkholderia*.

Multiplex real-time PCRs offers both practical (eg. throughput, limited handling) and scientific (eg quantification compared to a reference, internal controls) advantages. However, real-time PCR permits only limited multiplexing while we need to be able to rapidly screen for multiple pathogens (including viruses). Therefore, we are expanding our multiplexing capabilities by developing DNA microarrays as highly parallel diagnostic tools. In order to maintain the speed in the detection procedures, we are working with bead-based suspension arrays. In this microarray format, color-labeled nanobeads coated with probes enable multiplexing of 100 targets which can be rapidly measured by flow-cytometry. The liquid matrix permits rapid reaction kinetics and thus short hybridization times.

We will explore alternative assay formats and nucleic acid or signal amplification methods to examine the performance of DNA microarrays with respect to sensitivity, specificity and speed.

We have identified discriminating targets (phylogenetic as well as virulence markers) for the biothreat agents mentioned above and have used these for the design of short oligos for direct hybridization assays. In addition, we will explore the enhanced selectivity that can be achieved by using a solution-based chemistry involving a sequence-specific enzymatic reaction (ligase).

To achieve acceptable sensitivity and for labeling, we will amplify target DNA by multiplex PCR. When the number of targets to be amplified increases this approach may become impracticable, and therefore we will also explore the use of random amplification methods. In addition, the applicability of post-hybridization, on-chip signal amplification by using rolling-circle amplification will be investigated.

In parallel to the microarray development, methods for rapid and efficient DNA and RNA extraction and cleanup from diverse matrices and sample types are being developed and tested.

Occurrence and genomic diversity of *Bacillus anthracis* isolated in a wool-processing plant.

P. Wattiau¹, D. Fretin¹, S. R. Klee², M. Van Hessche¹, M. Ménart¹, G. Hanquet³, E. Kissling³, M. Poncin⁴, P. Butaye¹ and H. Imberechts¹

¹ Veterinary and Agrochemical Research Centre, Department of Bacteriology and Immunology, Brussels, Belgium

² Robert Koch Institute, Centre for Biological Safety, Berlin, Germany

³ Institute of Public Health, Department of Mycology, Brussels, Belgium

⁴ Occupational Medicine PROVIKMO, Belgium

Industrial anthrax, also known as “wool sorter’s disease”, was a serious threat in the 19th century when the wool industry was flourishing. In the U.S., apart from the 2001 attacks, the latest anthrax epidemic was reported in 1957 in a goat-hair processing mill in Manchester (New Hampshire). Today, industrial processing of wool and goat-hair has almost disappeared from Western industrialised countries. A very few number of plants subsist in regions with textile industrial history. In Belgium, an industrial plant performing middle-scale cleaning and disinfection of wool and goat hair has been discontinuously operating since 1890. In the present work, we investigated this plant for the presence of *Bacillus anthracis* in raw fibers, dust, wastewater and wastewater sludge. Living anthrax spores could be isolated from goat hair fibers, unprocessed wastewater and air dust collected from industrial filters. Forty three *B. anthracis* isolates picked-up randomly were analyzed by variable non-tandem repeats (VNTR) typing. All isolates belonged to VNTR cluster A4 and matched a few distinct subtypes, suggesting that contamination of goat hair batches by *B. anthracis* originates from multiple sources rather than from a single one. A serological survey conducted on the employees of the manufacture revealed that some workers had circulating antibodies directed against the anthrax Protective Antigen. Epidemiological assessment tentatively identified goat hair handling as the main risk factor. Since no case of human anthrax was ever reported within this population, it is evident that sub-infectious human contamination occurred.

This study allowed the “at risk” activities to be identified in the plant. Recommendations for effective measures were made to protect workers against anthrax contamination.

The Question; The Answer; And The Role of the “Black Box” – Molecular typing of *Brucella* isolates from Israel.

Sally J. Cutler¹ and Menachem Banai²

1. School of Health & Bioscience, University of East London, London, E15 4LZ, UK.

2. Kimron Veterinary Institute, P.O. Box 12 – Bet Dagan, 50250, Israel.

With the advent of diverse molecular tools and their application for identification and typing of Brucellae, we need to focus on the specific questions for which we need information in order to choose the most appropriate molecular technique to apply. This can even vary within a specific technique, as seen with multi locus variable repeat analysis (MLVA; also known as VNTR). A frequent first question is “what is the isolate (species or biovar)?”. This is traditionally achieved through lengthy microbiological biotyping with its associated problems of standardisation and laboratory acquired infection risks. Another major question is “what is the characteristic ‘signature’ or ‘type’ of the isolate facilitating outbreak investigations?” To this end, MLVA has been designed with two panels A and B, the first providing a molecular equivalent to biotyping; and a combined set of panels enabling the characterisation of the molecular signature of a particular isolate. Use of panel B alone should be avoided to prevent chance findings through homoplasmy. In order to provide a harmonised global scheme, a workshop funded through COST 845 facilitated training and dissemination of this technique to other nations.

We report the application of this technique to analyse isolates from Israel, including those from human and livestock origin and from different geographical locations. Whereas classical biotyping revealed limited differences, use of MLVA panel A, revealed all isolates to be *Brucella melitensis* biovar 2. This profile differed from that of the 16M *B. melitensis* vaccine strain. Results using panel A revealed all 20 isolates were totally conserved. Use of the combined panels A and B to provide the molecular “signatures” for these isolates revealed subtle differences between different groups isolates, but only with three of the variable regions examined. High resolution markers additionally showed mixed band sizes in multiple markers for one sample. Although markers can sometimes vary by a single unit up or down, this particular sample varied by two units in one example, thus we cannot exclude the possibility of mixed infection, a possibility that could have been overlooked using conventional methods.

In conclusion, we have shown the power of this approach for addressing both the question of identification, and furthermore, the ability of MLVA as an epidemiological typing tool for brucellosis.

Molecular Differentiation of the genus *Brucella* on the basis of virulence associated genes

J. Jacob, M.E.A. Mielke

Robert Koch-Institut, Nordufer 20, 13353 Berlin, Germany

The genus *Brucella* causes chronic infections in both humans and a variety of animal species. In a former publication, we characterized a spontaneous smooth small colony variant of *B. abortus* S19 which differs not only in growth rate but, most importantly, also in a less effective clearance from spleens and livers of experimentally infected mice. Using a differential display approach to analyze mRNA-derived cDNA, we identified a difference (Jacob, J. et.al., *Microbes and Infection* 8(2006) 363ff.) in the transcription of a gene coding for a formerly described galactoside transport ATP binding protein *mgIA*. In this work we found significant genetical differences within both *mgIA* and corresponding regulator genes by comparison of the homologous genome regions of *B.abortus* S19 and *B.neotomae* versus *B.melitensis* 16M as reference strain.

We sequenced the *mgIA* gene as well the flanking regions in *B.abortus* S19 and *B.neotomae* and compared the results versus genebank derived sequences from *B.melitensis* 16M (BMEII0981-BMEII0988).

Fig 1:

B.abortus S19 (Vaccine strain)	B.neotomae (Avirulent)	B. melitensis 16M (Virulent)
nirK, gene present, sequenced, not mutated	nirK, partly deleted (sequenced)	NIRK COPPER-CONTAINING NITRITE REDUCTASE PRECURSOR BMEII 0988
nirV, gene present, sequenced, not mutated	*nirV, deleted (sequenced), part of 2,2 kb deletion	NIRV PRECURSOR BMEII0987
*nnrA, gene present, sequenced, not mutated	*nnrA, partly deleted (sequenced), part of 2,2 kb deletion	*BMEII0986 nnrA, TRANSCRIPTION REGULATOR,
*Gene present, sequenced, not mutated	*Gene present, sequenced, not mutated	*BMEII0985 RIBITOL OPERON REPRESSOR
		BMEII0984 HYPOTHETICAL PROTEIN
Gene present, sequenced, not mutated	Gene present, sequenced, not mutated	BMEII0983 -GALACTOSE-BINDING PERIPLASMIC PROTEIN PRECURSOR ...
pseudogene <i>mgIA</i> present, sequenced, mutated, 24 bp deletion	* <i>mgIA</i> Gene present, sequenced, not mutated	*BMEII0982 GALACTOSIDE TRANSPORT ATP-BINDING PROTEIN MGLA
Not sequenced	Gene present, sequenced, not mutated	BMEII0981 XYLOSE TRANSPORT SYSTEM PERMEASE PROTEIN XYLH

* not mutated, * mutated

Within the genus of *Brucella*, the *mglA* gene has originally been described in *Brucella melitensis* 16M. In *B. abortus* genomes (strains S19A,B; 2308 and 9-941), it has been considered as a pseudogene because of a deletion of 24 basepairs in the *mglA* ORF when compared to the corresponding gene at position BMEII0982 in *B. melitensis*. This deletion was found not to be present in the avirulent *B. neotomae*. However, in case of *B. neotomae* the regulator genes (homologous to BMEII0985-0986) were found to be deleted. Summing up, in the highly virulent *B. melitensis* 16M both *mglA* and regulator genes are intact, in the vaccine strain *B. abortus* S19 *mglA* is modified, but not the regulator genes. In contrast, the avirulent *B. neotomae* possesses intact *mglA* but deleted regulator genes.

It is tempting to hypothesize that the genomic region around *mglA* is involved in modulation of virulence in *Brucella*. With respect to this we propose that the corresponding genome region within *brucella* species should be selected for use on an microarray for extensive testing of *brucella* libraries for genetical differences.

AFLP strategy for phylogenetic assignation of species

S. Panaiotov, V. Levterova, N. Brankova, T. Kantardjiev

National Center of Infectious and Parasitic Diseases, Janko Saksov 26, Sofia 1504, Bulgaria

Biochemical identification of new species has considerable limitations. Amplified fragment-length polymorphism (AFLP) is a whole genome typing technique based on selective amplification of site specific (restriction enzyme–digested) DNA fragments in order to create unique fingerprint for a particular genome. AFLP is utilized for typing of microbial organisms, genealogical studies among closely related species, quantification of genetic diversity within and among species, and phylogenetic studies. The power of the AFLP technique derives from the ability to quickly generate large numbers of specific marker fragments for any organism that could be used for phylogenetic assignment of unidentified species.

Objectives: To develop methodology that correctly assigns phylogenetic relatedness between known genetic patterns and unknown species under study.

Methods: We investigated the possibility to apply the AFLP technique as an identification tool for bacteria and fungi. More than 150 mycobacterial isolates, 80 fungi and other 120 bacterial species have been tested. BamHI, PstI, MboI, HindIII, HaeIII and XhoI restriction enzymes, appropriate adaptors for the restriction sites, primer variations and amplification conditions have been tested with the aim to identify characteristic AFLP species-specific patterns. AFLP fragments separation was performed on ALFexpress II sequencer and data elaboration with GelCompar II software.

Results: Best results for the AFLP method were obtained when genomic DNA is digested with PstI and BamHI restriction enzymes, synthetic adaptors are ligated by T4 ligase to the PstI and BamHI fragment sites, and fragments are specifically amplified with PstI and Cy5 labeled BamHI primers. We demonstrate that every species has unique AFLP pattern. Database of AFLP patterns has been created and in use for identification of new AFLP patterns.

Conclusions: At present commercially available AFLP kits use two restriction enzymes – EcoRI and MseI to digest DNA. The fragments are amplified by using A-, T-, G- C- or combinations for selective fragment amplification. As a result only the number of the amplified fragments is reduced and no additional polymorphic sites are found. For maximum discrimination the AFLP should be developed with respect to the genomic data. More restriction enzymes should be investigated to identify the best enzyme pair giving maximum valuable polymorphisms. AFLP analysis has been shown to be a valuable method for identifying micro(organisms). By establishing an AFLP pattern database one might be able to identify unusual isolates and on the basis of the specific genomic pattern to assign phylogenetically unidentified new species. To obtain good and reproducible AFLP profiles it is essential that DNA is isolated from fresh cultures and free of RNA. It was found that residual RNA molecules could seriously compromise PCR amplification. Residual non ligated

adaptors inhibit AFLP analysis. The comparison of different AFLP patterns could identify genus, species, and strains' specific bands. For phylogenetic studies and identification purposes, genus and strain specific bands are of major interest. For epidemiological strains' typing, of major interest are the highly polymorphic strain specific bands. AFLP analysis might prove to be a reliable tool for identification and typing of unidentified isolate(s) and assignment of phylogenetic position.

Molecular epidemiology of Swiss Bacillus anthracis, where do these strains come from?

Paola Pilo and Joachim Frey

Institute of Veterinary Bacteriology, University of Bern, Switzerland

Bacillus anthracis, the causative agent of anthrax, is a gram positive rod shaped bacteria. Its life cycle alternates germination and host infection with sporulation and survival in the environment. Spores are found in the soil and are spread worldwide. Anthrax is mostly an herbivore disease but all mammals can be infected. In humans, it is primarily a professional disease principally among breeders, veterinarians and wool processing factory workers.

B. anthracis is a genetically very homogeneous species and the most suitable technique to subtype these species is based on multi locus variable number of tandem repeats (VNTR) analysis (MLVA). Molecular genetics provides powerful tool to subtype and identify bacterial strains down to their original isolates. This permits extensive and precise molecular epidemiological analysis.

In Switzerland, cattle anthrax has been eradicated in the 1960s by strictly prohibiting dead animals or slaughtered waste to be buried and consequently burning carcasses from animals that died from diseases. However, between 1978 and 1981, small outbreaks of human anthrax occurred in a wool processing factory which processed synthetic fibers and goat wool imported from the Indian subcontinent.

We analyzed and compared Swiss isolates of B. anthracis from husbandry and industrial anthrax cases, collected between 1952 and 1981, with the published data from other studies using MLVA. We showed that isolates from cattle are autochthonous while industry isolates are imported strains due to the international Cashmere wool trade.

Development of a real-time PCR targeting IS711 gene for rapid detection of *Brucella* in samples

Lotfi Bounaadja¹, **David Albert**², Bruno Garin-Bastuji², Sylvie Poliak¹, Michel Zygmunt³, Benoît Chenais⁴

Departmental Laboratory of Sarthe, 128 rue de Beaugé, 72000 Le Mans, France¹

AFSSA, OIE/FAO Brucellosis Reference Laboratory, BP67, 94703 Maisons-Alfort, France²

INRA, Immunology and Infectious Pathology Laboratory, 37380 Nouzilly, France³

Genetics and Evolutionary Biology Laboratory, Maine University, Av. O. Messiaen, Le Mans, France⁴

Brucellosis is a disease caused by *Brucella*, a BSL 3 pathogen, Gram-negative, facultative intracellular bacteria that can infect a variety of warm-blooded animal including man. The genus *Brucella* comprises six species: *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Brucella ovis*, *Brucella canis*, *Brucella neotomae* and two new species recently published, *Brucella ceti* and *Brucella pinnipedialis*. A ninth species, *Brucella microti* was recently isolated from common vole *Microtus arvalis* (Paper in press).

Human Brucellosis is usually characterised by an intermittent influenza-like clinical pattern which may be severe and may be followed by chronic, intermittent relapses. The main manifestation in animal brucellosis are reproductive failure, i.e. abortion, orchitis and epididymitis in the male, and rarely arthritis. Persistent infection with shedding of *brucella* in reproductive and mammary secretion is common.

Diagnosis of *Brucella* infection can be obtained by bacteriological examinations and cultures but this process takes time and due to the zoonotic nature of most *Brucella* species, there is a potential hazard for laboratory personnel who must manipulate

The direct detection of *Brucella* can be obtained using molecular tools (PCR). Two mainly reported genes (IS711, BCSP31) are currently used in conventional PCR. In order to increase the sensitivity and the specificity of the direct detection of *Brucella* we have recently begun the development of a Real-Time PCR.

Three candidate genes (IS711, BCSP31, *per* gene) were selected. A panel of 71 non-*Brucella* strains and 8 *Brucella* reference strains were used in this study to evaluate the sensitivity and specificity of the assay.

BCSP31 and *per* genes gave the same level of sensitivity. Real-time PCR detection of *Brucella* sp. using the insertion sequence IS711 showed a higher sensitivity than the two others. Nevertheless, *per* gene was the most specific target since no cross-reactions were observed. *Ochrobactrum anthropi* (LMG 3306), *Listeria monocytogenes* (NCTC 7973) and *Rhizobium vitis* (LMG 8750) gave positive signals when BCSP31 primers and

probe were used. *Brucella* detection based on IS711 amplified *Streptococcus bovis*, *Serratia marcescens*, *brevibacillus*, *Rhizobium radiobacter* (LMG 140) and *Rhizobium vitis* (LMG 8750) DNA.

In order to assess first results, samples were collected from wild animals (wild boars), domestic animals (swine) and infected sheep and goat (ongoing studies).

It seems difficult to find a target with a strong sensitivity and a strong specificity. In PCR, reaction volume is very small, consequently, it's important to have a very sensitive assay. IS711 could be an interested target, however, the sensitivity and the specificity of this insertion sequence have to be evaluated with the recruiting of more samples from infected herds and on different matrices in order to be close to diagnosis conditions.

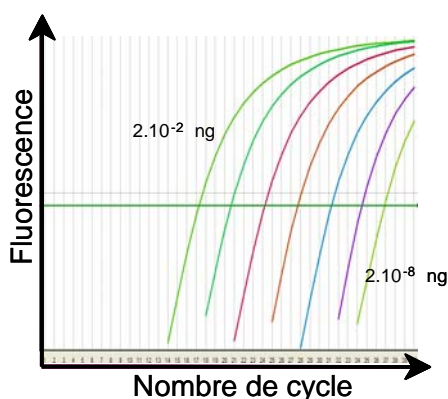


Figure 1 :

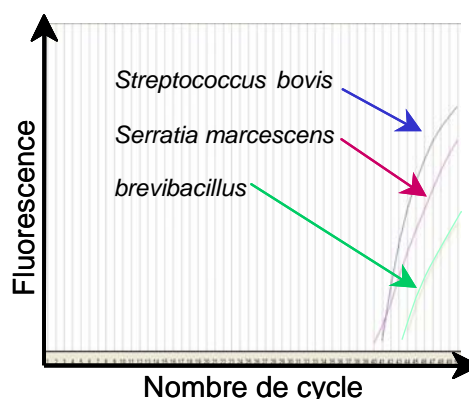


Figure 2

Amplification of insertion sequence IS711 with $2 \cdot 10^{-2}$ to $2 \cdot 10^{-8}$ of *Brucella ovis* DNA (figure 1) and specificity of BCSP31 detection (figure 2).

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Laboratory aspects of opportunistic fungal infections and the resistance to antifungal agents in immunocompromised patients

M. Nica*, T. Biolan*, E. Mozes*, A. Dascalu*, D. Caliga* , S. M. Ersoiu*, D. Duiculescu*,
E. Ceausu**, P. Calistru**, I. Apostol***

**Clinical Hospital for Infectious and Tropical Diseases Dr Victor Babes (SVB), Bucharest, Romania;*

***UMF Carol Davila; *** Dr Victor Babes Foundation*

Introduction: The opportunistic fungal infections has become a major medical issue, based on the increasing patient population at risk, potentially fatal conditions, difficulty in species identification of these fungi and the emergence of resistance to antifungal agents.

Objectives:

- 1 To study the incidence of invasive mycoses in patients admitted in SVB between Jan 2006-Oct 2007
- 2 To monitor the emergence of in vitro resistance of mycotic isolates to antifungal agents, especially azoles.

Material and method: The mycologic diagnoses was based on direct microscopy, isolation on specific media (Sabouraud agar–Chloramphenicol, CHROM-agar cultures) and automated system-BacT/ALERT. For identification we have used classical methods and automated methods (Vitek System and API system). The antifungal susceptibility testing was made using ATB FUNGUS 3 and E-test, on RPMI 1640 medium, in conditions similar to reference techniques NCCLS M27 A2/2003. Quality control strain: *Candida albicans* ATCC 90028.

Results: There was examined a total of 5980 clinical specimens from immunocompromised patients, admitted in SVB in this period and was isolated 98 infections of fungal etiology. There were identified 56 species of *Candida albicans*, 33 species of *Cryptococcus neoformans* and 9 species of *Aspergillus* spp (5/*A. niger*, 2/*A. fumigatus*, 2/*A. flavus*). Isolates of *C. albicans* was found to be resistant to 5-flucytosine (2/56), amphotericin B (4/56), fluconazole (10/56) and itraconazole (14/56); no isolates of *C. albicans* was resistant to voriconazole (MIC>4µg/ml) or caspofungin (MIC >1µg/ml). All isolates of *C. neoformans* and *Aspergillus* spp was sensitive to voriconazole and caspofungin.

Conclusions:

- 1 The highest frequent opportunistic fungal infection was candidiasis, followed by cryptococcosis and aspergillosis.
- 2 There is an emergence of in vitro resistance of fungal isolates to fluconazole, in immunosuppressed patients and there is need of active surveillance for crossed emergence of resistance to azoles. Voriconazole and caspofungin has excellent in vitro activity in systemic infections with opportunistic fungi.
- 3 The urgent need for introducing molecular diagnosis in identification of fungal infections in immunocompromised patients and for documentation of genetic bases of antifungal resistance.

Presentation of the CReSA institute

Dr. Xavier Abad

Centre de Recerca en Sanitat Animal (CReSA), Barcelona, Spain

The institute "Centre de Recerca en Sanitat Animal (CReSA)", an initiative of Universitat Autònoma de Barcelona (UAB) and Institut de Recerca i Tecnologia Agroalimentàries (IRTA), is focused on the research, technological development, training and education in the field of animal health. CReSA has defined objectives 1) to develop research programs within the field of animal health 2) to transfer the achieved scientific advances to the sector 3) to offer services within the field of research and development by arranging R+D programs 4) to advise agrifood companies and public administration and to provide technological support in the field of animal health and 5) to organize scientific and technical training programs

CReSA achieves these objectives with a team of over 60 expert scientists and technologically advanced biosafety level 2 laboratories and biosafety level 3 facilities. This biosafety units enable scientists to carry out research with pathogenic agents listed as diseases notifiable to the World Organization for Animal Health (OIE) like West Nile fever, Avian influenza and Newcastle's disease, Tuberculosis, Bluetongue etc. Scientific research in CReSA covers all the disciplines like Immunology, Virology, Molecular and cell biology, Bacteriology, Pathology/ anatomy Cell culture Parasitology and entomology. Management and researchers at CReSA are involved in a number of EU and National-funded projects and have experience in coordinating international and multidisciplinary projects. CReSA has facilities to accommodate and perform experimental infection experiments with smaller (mice, rats etc) and larger (cows, pigs, sheeps and goats etc) animals and poultry. Some of the ongoing projects in CReSA includes;

- Experimental infection with avian influenza virus in chicken
- New strategies for immunization against PRRS, flu, circovirus, classical and African swine fever
- Variability and characterization of field strains of Haemophilus parasuis
- Resistance and/or genetic susceptibility to the swine circovirus
- Variability of PRRSV strains isolated in Spain
- Epidemiology of swine flu in Spain
- Hepatitis E virus infection in pigs: diagnosis, epidemiology and pathogenesis
- Pathogenics, epidemiology and molecular biology of torque teno virus
- Identification of the Streptococcus suis iron and zinc uptake systems
- Composition of the diet, voluntary ingestion and intestinal health of piglets at weaning
- Infection of seronegative pigs with the Pestivirus isolates from chamoises
- Environmental persistence and immunopathogenics of the Avian influenza virus
- Molecular characterization of Gumboro's disease virus strains
- Identification of the proteins involved in divalent cation uptake in Salmonella enterica

- Isolation and characterization of bacteriophage of *Salmonella enterica* for their application as agents of biocontrol
- Nutritional strategies to reduce the incidence of necrotic enteritis in poultry production
- Risk of the generation of antimicrobial resistance with different medicinal dosages
- Epidemiological and immunological aspects of neosporosis in cattle
- Study of the *Culicoides* vectors of the Bluetongue disease in Spain
- Pathogenesis of prionic and neurodegenerative diseases